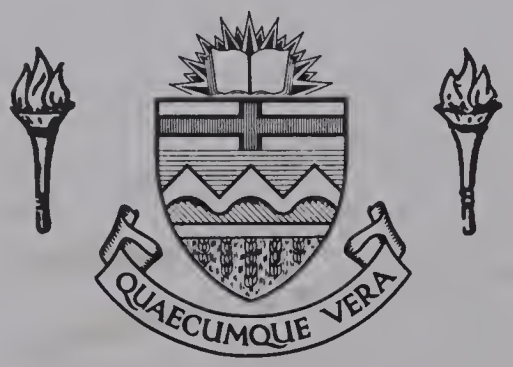

THE UNIVERSITY OF ALBERTA

BIOCHEMICAL COMPOSITION OF F-PILI

by

JOHN GABRIEL DONELIAN

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "BIOCHEMICAL COMPOSITION OF F-PILI", submitted by John G. Donelian in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Investigations have been performed to determine the biochemical composition of *E. coli* HB11 F^+ F-pili.

To quantitate relative amounts of F-pili in bacterial and cell-free suspension, an assay procedure has been developed which measures varying numbers of these structures in terms of "cell-equivalents" of *E. coli* HB11 F^+ F-pili. *E. coli* HB11 F^+ was chosen as a reference bacterium for this study since it possesses only one F-pilus per cell (average) and because its only surface appendage is the F-pilus.

Removal of F-pili from HB11 bacteria into the supernatant phase of whole cultures has been achieved by a combination of mechanical shearing (blending) of host cells and spontaneous (natural) release of F-pili during the cell growth process as versus blending or natural release alone. Blending of HB11 cultures (5.5×10^8 cells/ml) at 2500 r.p.m. for 30 seconds converted 40 to 45% of all available F-pili into a relatively unfractured, cell-free state whereas in untreated cultures (5.5×10^8 cells/ml) some 25% of all available F-pili were similarly converted into the cell-free state by natural release. Where both methods were used in combination, yields were essentially cumulative, with 65 to 70% of all available F-pili being found in the cell-free state in the supernatant phase of whole, blended cultures.

Large scale production of "pure" F-pili (utilizing 6 mM 2-mercaptoethanol throughout as an F-pili stabilizing agent) has been achieved by growth of HB11 bacteria in 30 litre volumes, mechanical removal of F-pili from host cells by a continuous-flow blending process,

concentration of cell-free F-pili from culture supernatants by acid (pH 4) precipitation, partial removal of cellular contaminants by extensive alkaline dialysis, and final purification of F-pili by CsCl density gradient centrifugation. Such preparations were found, by isotope dilution and SDS-polyacrylamide gel electrophoretic analyses, to contain only trace levels of cellular contamination and were judged suitably pure to undergo chemical analysis.

Chemical analysis of "pure" F-pili revealed that carbohydrate, protein and organic phosphate are present in the structures examined, with no detectable level of nucleic acid or lipid being found. The major carbohydrate moiety in F-pili was judged to be either pentose or 6-deoxyhexose in nature, and a lesser amount of what appeared to be galactose was present as a second minor component. Both major and minor sugar components were shown to be reducing sugars. Ribose and 2-deoxyribose were not present in the F-pili material examined. The protein F-pilin was found to contain no unusual amino acids, although 22% of its amino acid composition was collectively contributed by aspartic and glutamic acids. F-pilin was shown to have an average molecular weight of 8×10^4 daltons. The small amount of organic phosphate found was shown not to be phospholipid or nucleic acid in nature, and may be protein-linked.

"The secrets of Nature reveal themselves more readily when tormented by Art than when left to their own courses."

Francis Bacon, 1620.

DEDICATION

To My Wife

Who makes life something truly worthy of being loved.

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LIST OF ABBREVIATIONS

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
RTF	resistance transfer factor
col I	colicinogenic factor I
F factor	fertility factor
Hfr	high frequency recombinant
SDS	sodium dodecyl (lauryl) sulfate
met	methionine
λ	temperate phage λ
Sm^s	streptomycin sensitive
Lac	the lactose operon
TMM	Tris (hydroxymethyl) aminomethane maleic acid minimal salts medium
TSB	trypticase soy broth
NaDS	sodium dextran sulfate 500
PEG	polyethylene glycol 6000
2-ME	2-mercaptoethanol
mCi	millicurie - 2.22×10^9 disintegrations per minute
PFU	plaque forming units
SSC	standard saline citrate 0.15 M NaCl, 0.015 M sodium citrate; pH 7.0
g	centrifugal force relative to gravity
c.p.m.	counts per minute
TCA	trichloroacetic acid
EDTA	ethylenediaminetetraacetic acid

Tris	Tris (hydroxymethyl) aminomethane
TEMED	N,N,N',N'-tetramethylethylenediamine
mA or mAmps	milliamperes
p/c	phage particle to bacterial cell ratio (numerical)
r.p.m.	revolutions per minute
CE	cell-equivalents *E. coli* HB11 F^+ F-pili
dalton	1.66024×10^{-24} gram
rRNA	ribosomal ribonucleic acid
RCF	relative centrifugal force times gravity
mol. wt.	molecular weight
m.o.i.	multiplicity of infection
A_{650} A_{550}	light absorbancy of a solution in a 1 cm light path at 650 mμ (550 mμ)

All temperatures were expressed in degrees Centigrade except when specified otherwise.

CHAPTER I

INTRODUCTION

It has been known for two decades that gram-negative bacteria possess two distinct types of filamentous appendages originating at the cell surface—flagella and fimbriae (pili) (Brinton et al., 1954; Duguid et al., 1955; Houwink and Van Iterson, 1950; Maccacaro and Hayes, 1961; Brinton, 1959). Flagella are the organelles responsible for cellular motion, and their chemistry, genetics and morphology are described in a recent review by Iino (1969). Whereas flagella are commonly considered to consist entirely of the protein flagellin in most bacterial strains, and are structurally identical in many non-related strains of bacteria (Iino, 1969), two or more functional types of pili of separate and distinct structure can be produced by a single gram-negative bacterium (Novotny et al., 1969a; Novick, 1969), each of which differs morphologically from flagella (Houwink and Van Iterson, 1950; Valentine et al., 1969).

Bacterial pili can be classified, on the basis of structure and function, into two major groups—types I through V pili, and "sex" pili. Types I through V pili are short, rigid, proteinaceous structures present on both male and female gram-negative bacteria. Although the function of these pili is completely unknown at present, they may possibly serve to stabilize mating pairs during bacterial conjugation (Brinton, 1965; Gemski, 1964). Sex pili, which, in enteric bacteria, are specialized receptors required for genetic transfer during bacterial mating, and which are used also as adsorption sites by male-specific phages (Novick, 1969), are genetically determined by transmissible

plasmids called sex factors (a plasmid being an "extrachromosomal" DNA element existing as a stably inherited component of the cell genome when physically separate from the chromosome; Novick, 1969). Several types of sex factors are known. One type of sex factor, whose function is to render their hosts resistant to antibacterial agents such as antibiotics, and, at the same time, enable them to transmit resistance to other bacteria, are termed resistance transfer (RTF) factors. The recipient of an RTF factor becomes both drug-resistant and a genetic donor at the same time (Meynell, Meynell and Datta, 1968). A second class of sex factor, the colicinogenic factors, are those which genetically control the synthesis of certain antibiotics of the bacteriocin type which are called colicins (Jacob and Wollman, 1961; Nomura, 1967). The term bacteriocins, as coined by Jacob, Lwoff, Siminovitch and Wollman in 1953, applies to antibiotic substances which are produced by certain strains of bacteria, and that are lethal to other strains of the same species. Type I pili, produced by the colicinogenic I factor (Col I), are an example of this type of "sex" pilus. A third class of sex factor, the fertility or "F" factor, is the genetic element responsible for determining a bacterial donor type. Lack of the F factor in a bacterial strain results in the F^- recipient (female) donor type, whereas the presence of this genetic sexual determinant defines the male donor type. An F^+ donor is a male bacterium harboring the F factor as an entity physically separate from the host genome. Likewise, an Hfr donor is a male cell containing the F factor as an integrated part of the bacterial chromosome.

The role of F-pili in promoting viral infection of male donor types of E. coli K12 bacteria by a small RNA-containing "male-specific"

bacteriophage (R17), was first investigated by Crawford and Gesteland in 1964. These researchers observed by electron microscopy that when phage R17 was added to a culture of male cells, the phage particles adsorbed selectively to certain pili attached to the cell surface. No affinity for the pili of female (F^-) cells was demonstrated, nor were these cells infected by the virus. This now classic observation by Crawford and Gesteland was the first indication that F-pili play an intimate role in the transport of the viral genome into the bacterium by some unknown means.

In 1964, Brinton, Gemski and Carnahan confirmed these findings, naming the filaments to which the phage adsorbed, F-pili, to distinguish them from other types of pili synthesized by both male and female strains. They found that only one or two of the large number of pili which could occur on the cell surface were F-pili, and that these pili were genetically determined by the fertility (F) factor contained in the plasmid DNA of E. coli. Moreover, they showed that F-pili were mandatory for bacterial mating to occur between male and female E. coli bacteria, and that deleting the F factor from male mating types with acridine orange produced phenotypically and genotypically female bacteria, indicated by the physical absence of any F-pili in the resultant cells. Finally, if the F factor was introduced into F^- cells by bacterial conjugation, fertility or "maleness", as characterized by the presence of F-pili, was again observable (Brinton et al., 1964; Brinton and Baron, 1960).

In 1965, Brinton introduced his "F-pili conduction model", in which he proposed that the F-pilus might serve as the "conduction tube" or bridge for the transfer of nucleic acid into and out of the bacterial

cell across the wall-membrane barrier during viral infection and bacterial conjugation. This model was based on the observations that the dense central core, seen in negatively-stained preparations of F-pili, was of sufficient dimensions to accommodate a single-stranded RNA or double-stranded DNA molecule. Further, the presence of F-pili correlated well to competence of cells in the mating function and in supporting infection by male-specific phages (Brinton, 1965).

A strikingly different model for F-pili function has recently been proposed by Marvin and Hohn (1969). This "F-pili retraction model" is founded on the observation that cell-associated F-pili disappear completely from log-phase cultures of E. coli when infected with Ff, a filamentous DNA phage which attaches only to the distal tip of the F-pilus. As this disappearance, with time, of F-pili from the cell surface parallels (a) a loss of RNA phage adsorption sites per cell, and (b) the appearance of bacteria infected with Ff, the authors proposed that the F-pilus may retract into the cell. According to this model, moreover, adsorption of RNA phage to the side, or F^- cells to the tip of the F-pilus also mediates retraction of the pilus. In the retracted position phage then inject their nucleic acid into the host, or donor and recipient cells form a conjugation bridge.

The F-pili "conduction" and "retraction" models both seem to be supported by the recent findings of Ou and Anderson (1970), who noted that not only do F-pili tend to draw mating pairs together to create a more effective union (thus indicating that the F-pilus retracts into either the donor or recipient cell during conjugation), but also that F-pili are involved in the direct transfer of chromosomes and F factor between Hfr and F^- bacteria. It has, however, now been established

that this phenomenon most likely does not occur in the case of RNA phage penetration (Krahn, 1971). Thus, whether the two models are mutually exclusive, i.e., that all nucleic acid transport via F-pili proceeds by only one mechanism, or not, is presently open to question. Although it has been established that only one of the three types of nucleic acid (i.e., phage RNA or DNA, host DNA) may be transferred by a given pilus at any given time (Knolle, 1967; Ippen and Valentine, 1967; Novotny et al., 1968), it is quite conceivable that they occur by entirely different mechanisms (Krahn, 1971).

F-pili are shown, by electron microscopy, to be tubular structures with an average diameter of 85 Å and an average inner core diameter of 20 Å (Brinton, 1965). They have been observed (Valentine et al., 1969) to vary in length from 0.1 μ to 20 μ and have, according to Ippen (1967), a mean buoyant density in CsCl of 1.197 gm/cm^3. Sucrose gradient sedimentation analyses of F-pili show that they are very heterogeneous in length (Valentine et al., 1969; Danziger, 1968; Wendt et al., 1966) presumably because of spontaneous breakage during growth and sedimentation of whole cells. No one has yet been able to provide evidence supporting a direct chemical association of nucleic acid with F-pili, although much indirect functional evidence is available supporting this view (see Brinton and Beer, 1967).

F-pili, upon exposure to organic solvents such as chloroform, benzene and CCl_4, were found to disintegrate rapidly (Wendt et al., 1966) and exposure to 80° heat in aqueous solution for 4 minutes caused 90% of the phage-adsorbing capacity of experimental F-pili to be destroyed (Valentine et al., 1969). Inuzuka et al. (1969) have shown that the anionic detergent sodium dodecyl sulfate causes deletion of

the F factor from Hfr cells by partial lysis of the bacterial membrane, presumably in the region where the F factor in the bacterial chromosome is attached to the cytoplasmic membrane (Inuzuka et al., 1969; Cuzin and Jacob, 1965; Jacob et al., 1965; Jacob et al., 1963). Bayer (1968c) has shown that the F-pilus appears morphologically as an extension of a cell wall - membrane complex associated with specialized receptors for male-specific phages.

Brinton (1965) has reported F-pili to be composed entirely of protein, a finding which is supported by the fact that they are digested by the proteolytic enzymes papain, trypsin, chymotrypsin and pepsin (Brinton and Beer, 1967). It is of interest to note that common (Type I) pili are reported (Brinton and Beer, 1967) not to be degraded by trypsin, again showing differences in structure between these two pili classes (Valentine et al., 1969).

The antigenic properties of F-pili were first reported by the Ørskovs (1960), who found a male antigen termed f^+ associated with male E. coli strains. This was confirmed by Ishibashi (1967), who showed that male-specific antibody adsorbed to the F-pili of E. coli K12 JE2218 (F^+); pili of F^- strains (type I) showed no agglutinating ability with the anti- f^+ serum. Ishibashi concluded that the F-pilus was the structure responsible for f^+ antigen activity (Valentine et al., 1969).

The foregoing is a brief summary of what is currently known about the biological, physical and immunological properties of F-pili. It is evident that although some preliminary efforts have been made to characterize F-pili, the information available is, for the most part, physiological, and no one yet has reported an in-depth study of the

physical or chemical properties of F-pili. For this reason, the elucidation, in part, of the physico-chemical nature of F-pili was chosen as the subject of this study. This thesis summarizes

(a) attempts to develop a means of assaying F-pili quantitatively,

(b) attempts to develop a method to isolate and purify F-pili, and

(c) the results of experiments designed to reveal the chemical nature of these structures.

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

(1) Bacteria and Bacteriophage

(a) Bacteriophage

The RNA phage R17 originally isolated by Paranchych and Graham (1962) was used throughout the studies described in this thesis.

(b) Bacteria

The following is a list of the strains of *Escherichia coli* employed:

Strain	Genotype	Source
E. coli K12	Hfr_1 met^- (λ^+)	A. Garen
E. coli B/r HB11	F^+ lac^+/lac^- Sm^s	C.C. Brinton, Jr.
E. coli B/r HB11	F^- lac^+/lac^- Sm^s	Derived by selecting an R17 resistant HB11 strain

E. coli HB11 was used throughout this work for the preparation of F-pili. This strain of *E. coli* was used as its only surface appendage is the F-pilus.

All strains of *E. coli* were maintained on hard agar plates for routine use. Permanent stocks were stored on hard agar slants in wax-sealed vials.

Aseptic technique was observed in all procedures involving the handling of phage or bacteria.

(2) Bacterial Culture Media

(a) Tris-(hydroxymethyl)-amino methane (Tris) maleate synthetic medium (TMM)

The basic TMM growth medium contained the following: 50 mM Tris; 50 mM maleic acid; 43 mM NaCl, 27 mM KCl, 19 mM NH_4Cl, 1 mM Na_2HPO_4, 1 mM Na_2SO_4, pH 7.4.

The above solution was prepared from a 10 X concentrate and autoclaved at 121° for 15 minutes under a steam pressure of 20 lbs/in^2.

Complete TMM (CTMM) medium was prepared by combining the sterile components listed in the following proportions:

Basic TMM	886 ml
5% (w/v) Casamino acids (Difco Laboratories)	24 ml
50% (w/v) D-glucose	10 ml
0.25% (w/v) L-methionine	20 ml
0.50 M $MgCl_2$	10 ml
20mg% (w/v) D-Biotin in TMM	50 ml

(b) Various TMM-based media

(i) Lactose TMM medium. This medium, used solely for culturing E. coli HB11, contained 50% α-lactose in place of D-glucose as the carbon source.

(ii) Low-phosphate CTMM. This medium, used in the preparation of ^{32}P-labeled R17 crude lysates, contained 0.5 mM Na_2HPO_4 as a minimal phosphate source.

(c) Trypticase Soy Broth (TSB)

Trypticase Soy Broth (Baltimore Biological Laboratories)	15.0 gm/l

NaCl	8.0 gm/l

Autoclaved solutions had a final pH of 7.2 - 7.3.

(d) Z Broth (Novotny et al., 1968)

Bacto Tryptone (Difco Laboratories)	10.0 gm/l
Yeast Extract (Difco Laboratories)	1.0 gm/l
NaCl	8.0 gm/l
D-glucose	1.0 gm/l
$CaCl_2$	0.44 gm/l

Autoclaved solutions had a final pH of 7.2 - 7.3.

(e) Hard Agar

Trypticase soy broth	30.0 gm/l
Bacto-Agar (Difco Laboratories)	15.0 gm/l

After dissolving, the solution was autoclaved and dispensed while warm into disposable Petri dishes (Falcon Laboratory Supplies).

(f) Top Agar

Top agar was prepared as above except that the final agar concentration was 10.5 gm/l. Sterile agar was stored in 50 ml volumes at 4° until used. At this time, the agar was melted in a boiling water bath, dispensed in 1.5 ml aliquots into sterile culture tubes, and maintained in the liquid state until used by incubation in a 50° water bath.

(3) Bacteria and Phage Diluent

All dilution of bacteria or phage to be plated was done in a sterile solution composed of 0.9% (w/v) NaCl, 5 mM $MgCl_2$, and 5 mg% bovine serum albumin (Sigma Chemical Co.). Diluent was dispensed

in 10 ml volumes into sterile dilution tubes (20 mm) and stored at room temperature until used.

(4) Chemicals, Enzymes and Reagents

Tris, maleic acid, unlabeled amino acids (L series), adenosine, deoxyadenosine, sodium dextran sulfate 500 (NaDS), egg-white lysozyme, D-biotin and bovine serum albumin were purchased from Sigma Chemical Co. Polyethylene glycol 6000 (PEG) was supplied by J.T. Baker and Co., as were 85% phosphoric acid and 91% formic acid.

Ultra-pure urea and standard electrophoretic proteins were purchased from Mann Research Laboratories. Dodecyl sodium sulfate from Matheson, Coleman and Bell was used without further purification. N,N,N',N'-tetramethylethylenediamine (TEMED) and 2-mercaptoethanol (2-ME) were supplied by Eastman Organic Chemicals. All other reagents required for polyacrylamide gel electrophoresis were from Canal Industries Corp.

Trichlorotrifluoroethane was purchased from Calbiochem. Orcinol was supplied by the British Drug Houses Ltd. Ultra-pure silver nitrate was purchased from Engelhard Industries of Canada, Ltd. Aniline oil was from Merck and Co.

Fisher Chemical Corp. furnished pyridine, D-glucose, D-galactose, D-ribose, D-xylose, D-arabinose, sucrose, α-lactose, diphenylamine, toluene, all concentrated inorganic acids (with exceptions noted), formaldehyde, acetone, chloroform, all alcohols and inorganic salts (with exceptions noted). Whatman No. 1 chromatography paper was supplied by Canadian Laboratory Supplies Ltd.

L-fucose, D-lyxose and L-rhamnose were the kind gift of Dr. R.U. Lemieux, Department of Chemistry, University of Alberta, Edmonton 7, Alberta, Canada.

(5) Radioactive Materials

Radioactive precursors for the preparation of labeled phage R17 and labeled E. coli HB11 F^- bacteria were obtained from the following sources: ^{32}P (inorganic phosphate) in 0.1 N HCl, Charles E. Frosst & Co.; ^{14}C uniformly labeled D-glucose (freeze-dried solid, 3.0 mCi/mM), Amersham/Searle Corp.

B. ROUTINE PROCEDURES

(1) Culture of Bacterial Cells

Experimental cultures were grown at 37° in a rotary shaking water bath from a 1/100 dilution of an overnight E. coli culture. To achieve maximum aeration and avoid excessive breakage of F-pili, shallow cultures (generally 20% of the flask volume) were shaken at a speed of 125 rpm in baffled culture flasks (Bellco Glass Co.). Under these conditions cultures generally reached a cell density of 2×10^8 cells/ml at about three hours after inoculation.

Preparation of large volume cultures is described later in the text.

(2) Determination of Phage Concentration and Bacterial Cell Density

(a) Bacterial cell density

The density of viable E. coli cells in all TMM-based media was determined from a standard curve constructed by plotting the absorbancy at 650 mμ of a 1.0 ml volume of culture vs. the viable cell counts. Viable cell counts were determined by plating an appropriate dilution of the culture on hard agar plates and incubating overnight at 37°.

(b) Phage concentration

The actual number of phage particles in a phage solution were

determined from optical density measurements with a Beckman spectrophotometer, using 7.66 absorbancy units/mg/ml/cm as the extinction coefficient at 260 mμ (Gesteland and Boedtker, 1964). Assuming a molecular mass of 3.6×10^6 daltons per phage particle, as reported by the foregoing workers, one absorbancy unit was calculated to contain 2.18×10^{13} phage particles.

The concentration of infectious phage (PFU/ml) in purified phage preparations was ascertained by the plaque assay described later in this chapter.

(3) Phage Preparation and Purification

(a) Preparation of crude lysates

Cultures of E. coli K12 Hfr were grown at 37° in CTMM medium to a cell density of 4×10^8 bacteria/ml, then infected by the addition of purified phage R17 at a multiplicity of infection (m.o.i.) of 40 PFU's/cell. After allowing several minutes for phage adsorption, incubation was continued with vigorous shaking (150 rpm) for 3-4 hrs at which time partial cell lysis was observed. Cell lysis was completed by adding lysozyme (66 mg/l) and chloroform (3.3 ml/l) followed by a further incubation at 37° for 30 minutes. The final yield of infectious phage in crude lysates varied from 8×10^{11} - 1.2×10^{12} PFU/ml.

(b) Concentration and purification of phage from crude lysates

The technique used to purify phage R17 was a modification of the liquid two-phase polymer method of Albertsson (1967). Following the addition of 71.50 gm polyethylene glycol (PEG), 2.06 mg sodium dextran (NaDS), and 18.00 gm NaCl per liter of crude lysate, the mixture was shaken until thoroughly homogeneous and stored at 4° for 24 hrs to

allow phase formation. At this time, most of the top PEG phase (comprising 90-95% of the total volume) was removed by aspiration, care being taken not to disturb the interphase. The lower NaDS phase and the interphase material containing cell debris and the highly concentrated phage were then centrifuged at 480 x g for 10 min to effect complete phase separation and the lower NaDS phase was then removed through a Pasteur pipette introduced into the bottom of the centrifuge tube. Phage was removed from the contaminating cell debris by extracting the interphase material with five equal volumes of 0.15 M NaCl buffered with 0.015 M sodium citrate, pH 7.0 (SSC), each time centrifuging out the cell debris at 10,000 x g for 10 minutes. Following exhaustive dialysis against SSC (4°), the phage was pelleted by centrifugation at 70,000 x g for 4 hrs in a Spinco #30 rotor and finally resuspended in 3.0 ml of sterile trypticase soy broth (TSB) in preparation for banding in a CsCl density gradient.

(c) CsCl banding of partially purified phage R17

As a final purification step, partially purified phage preparations were banded in CsCl density gradients. This was accomplished by adjusting the buoyant density of the phage solution to 1.46 gm/cm^3 by the addition of solid CsCl, and centrifuging at 105,000 x g for 40 hrs in a Spinco SW 50.1 rotor at 4°. After piercing the bottom of the tube, the contents of the gradient were collected into a series of tubes in 12 drop portions and the fractions containing the band of infectious phage were pooled and dialyzed against SSC at 4° to remove CsCl. Figure 2.1 illustrates the radioactivity and infectivity profile of a phage preparation labeled with ^{32}P. The fractions pooled in this case were fractions 10 - 15, since these six fractions contained more than

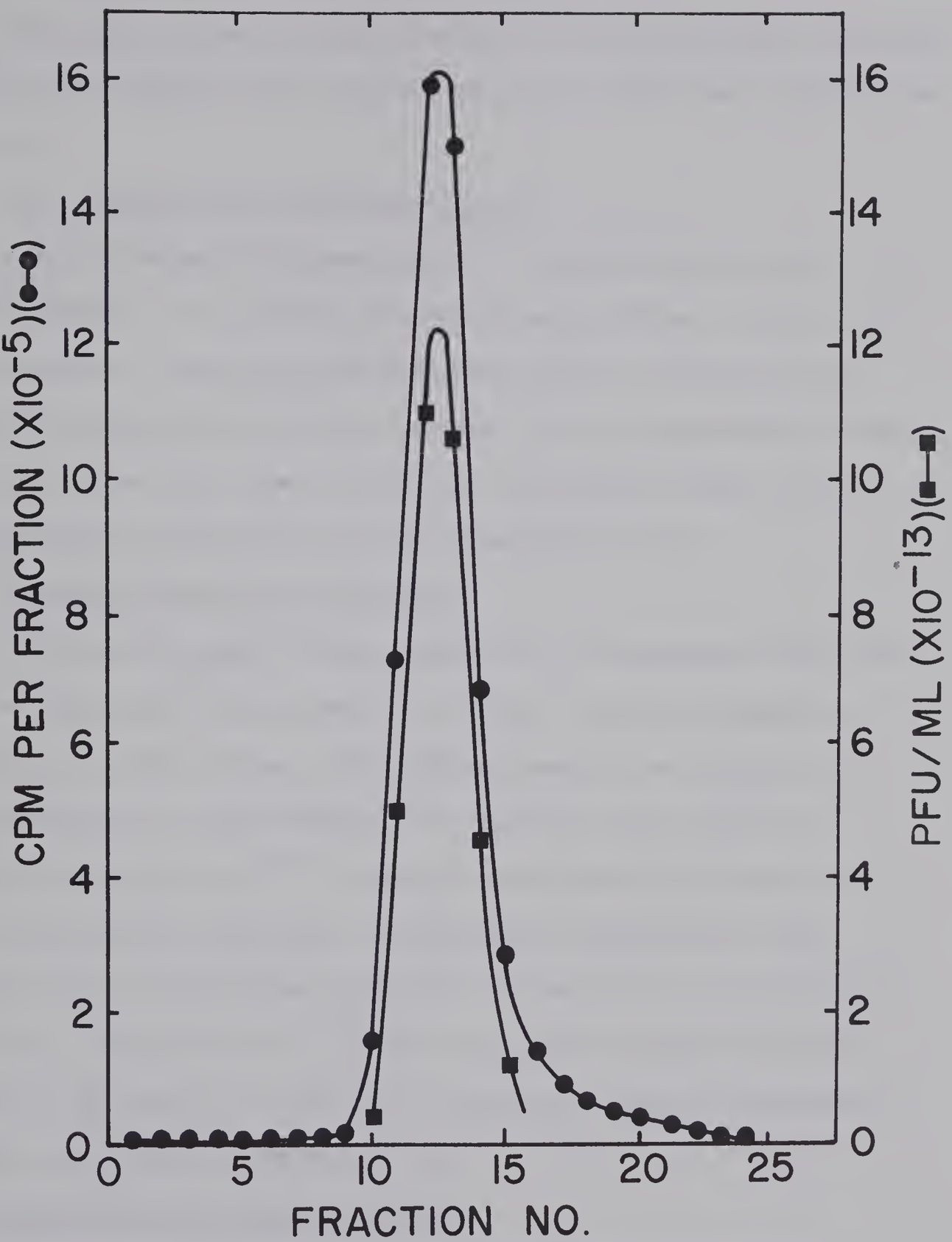


Figure 2.1. Cesium chloride density gradient centrifugation of ^{32}P-labeled phage R17.

The experimental details are given in the text.

90% of the total infectivity in the gradient.

Recovery of phage originally present in the crude lysates (measured as PFU) was normally about 90% while the particle/PFU ratio ranged from 5 to 12.

(d) Storage of purified phage stocks

Purified phage stocks were stored in concentrated solutions (1 - 4 x 10^{14} PFU/ml) at 4° in SSC containing 15 mg/ml TSB and a drop or two of chloroform. The latter additions were found to stabilize phage viability and prevented bacterial growth in the storage medium. Phage could be stored under these conditions for periods of time in excess of 6 months without appreciable loss of phage infectivity.

(4) Radioactive Labeling of Phage R17

Cultures of E. coli K12 Hfr_1 adapted to low phosphate CTMM medium by overnight culture were grown in 1 litre of fresh low phosphate medium at 37° and infected with purified phage R17 as described for the preparation of crude lysates. At 10 minutes post-infection, a neutralized solution of ^{32}P inorganic phosphate (15 mCi) was added and incubation was continued. Purified phage obtained from such crude lysates generally had a specific radioactivity of 4 x 10^{-7} cpm/particle. About 99% of the ^{32}P-label in purified phage was soluble in hot 5% TCA but not in cold 5% TCA, indicating that it was present as RNA rather than contaminating $^{32}P_i$.

(5) Plaque Assay for Infectious Phage

Determination of infectious phage concentrations was carried out by mixing 1.0 ml of a diluted phage sample and 0.2 ml of seed culture (approximately 4 x 10^8 bacteria/ml) with 2.0 ml of liquified top agar, followed by pouring the entire contents onto hard agar in a

Petri dish (Falcon Laboratories). After allowing the top agar to harden, the plates were inverted and incubated at 37° overnight before scoring for plaques.

C. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS TECHNIQUES

(1) Solubilization of F-Pili Protein

F-pili protein was prepared for electrophoresis by a method similar to that of Viñuela *et al*. (1967). Pure pili in pelleted form (pH 4.0) were resuspended in 0.10 M Tris-HCl, 10 mM EDTA, 0.14 M 2-ME, pH 8.4 in a total volume of 10.0 ml. This pili suspension was then shaken for 3 minutes with 4.0 ml of redistilled phenol saturated with 0.10 M Tris-HCl, 10 mM EDTA, 0.14 M 2-ME, pH 8.4, and centrifuged at 4,000 x g for 10 minutes to separate the phases. The extraction procedure was repeated 2 times, and the phenol phases plus interphase protein pooled. The phenol-protein mixture was dialyzed, with stirring, against 80 volumes of 0.10 M acetic acid and 0.14 M 2-ME at 25°. The dialysis buffer was changed every 2 hours until the phenol phase (with solubilized protein) was reduced to a volume of approximately 0.50 ml. The aqueous phase was removed and the same dialysis bag containing the phenol phase was resealed tightly and dialyzed 12 hours against 125 volumes of 9.0 M urea, 0.05 M $HC_2H_3O_2$ and 0.14 M 2-ME. The dialysis bag was then transferred into 100 volumes of 8.6 M urea, 0.01 M EDTA, 0.14 M 2-ME, 0.10 M Tris-HCl, pH 8.4, and dialyzed for 2 hours with vigorous mixing. N_2 gas was bubbled through the dialysis solution at this step. The dialysis bag was then transferred into 500 volumes of 0.01 M sodium phosphate, 0.1% SDS, 0.14 M 2-ME, pH 7.2, and dialyzed for 24 hours with one change of dialysis buffer. The sample, prepared as above, was used

directly for electrophoresis.

(2) Polyacrylamide Gel Electrophoresis

The gel solutions contained 10% (w/v) acrylamide, 0.05 M sodium phosphate, pH 7.1, 0.1% (w/v) SDS, 0.27% (w/v) N,N'-bismethylene acrylamide, 0.075% (w/v) TEMED, and 0.075% (w/v) ammonium persulfate (catalyst). After addition of catalyst, the gel solution was filtered through a Millipore type HA 0.65 μ filter and de-aerated under vacuum. Columns were formed by pouring 1.0 ml of solution into a coated glass tube (6.3 x 0.5 cm inner diameter), layering on 1 ml of water and allowing polymerization to occur at room temperature. After 20 - 30 min, the water layer was drawn off and the columns inserted into the assembly. Samples (0.2 ml) of the pili protein were mixed 1:1 with 60% glycerol and applied to the column by layering underneath the electrophoresis buffer (0.1% SDS in 0.1 M sodium phosphate, pH 7.1). Electrophoresis was carried out at room temperature under a current of 5 mAmps/tube, supplied by a Savant regulated power supply (Model HV 100 TC, Savant Inst. Inc., Hicksville, N.Y.), for 2.5 - 3 hours. After electrophoresis, the gels were removed and stained for 1 hour at 37° in Coomassie Blue stain prepared by dissolving 250 mg Coomassie Blue in 45.5 ml distilled-deionized water, 45.5 ml absolute methanol and 9.0 ml glacial acetic acid. The gels were then rinsed several times with 7% acetic acid and destained electrophoretically against 7% acetic acid at 12 mAmps/tube. Completed gels were stored in 7% acetic acid at room temperature.

(3) Polyacrylamide Gel Scanning

The electrophoretic gels were analyzed for protein bands by scanning in a Gilford Model 240 spectrophotometer equipped with gel

scanning adaptor. Settings were: absorbance, 540 mμ; ratio, 0.25; scanning speed, 2.0 cm/min; recorder speed, high; slit width, 0.05 x 2.36 mm.

(4) Molecular Weight Estimations of F-Pili Protein on Polyacrylamide Gel

Molecular weight estimations of F-pili protein were performed according to the method of Shapiro, Viñuela and Maizel (1967); standard electrophoretic proteins included bovine serum albumin (mol. wt. = 67,000 gm/mole), ovalbumin (mol. wt. = 45,000 gm/mole and 90,000 gm/mole dimer), and myoglobin (mol. wt. = 17,800 gm/mole).

D. CsCl ISOPYCNIC DENSITY GRADIENT ANALYSIS OF F-PILI

Aqueous samples (4.0 to 4.5 ml) of F-pili suspensions were made 1.8 M with respect to CsCl (ρ = 1.240 gm/cm^3 @ 25° by refractive index), placed into sealed rotor tubes (Spinco SW 50.1) and centrifuged at 105,000 x g for 40 hours in a Beckman Model L preparative ultracentrifuge (chamber temperature = 4°). Following centrifugation, the sample tubes were carefully placed into a gradient fractionating device (Buchler Inst. Corp., Fort Lee, N.J.) and secured tightly by a threaded screw cap. The bottom of the tube was then punctured and fractions collected dropwise into a series of tubes or onto mounted filter discs.

E. RADIOASSAY BY THE FILTER DISC METHOD

Samples (50 - 200 μl) to be assayed for TCA-insoluble radioactivity were transferred by pipette to pencil-marked 3MM Whatman filter discs (2.1 cm diameter) impaled onto a styrofoam base with steel pins, and processed by the scheme below (Mans & Novelli, 1960).

(1) Hot TCA Insoluble Products

Table 2.1

Preparation of Filter Discs for Radioactivity Measurement

Step	Time	Wash Medium	Temperature
1	30 min	10% TCA	4°
2	5 min	5% TCA	4°
3	45 min	5% TCA	90°
4	15 min	5% TCA	4°
5	15 min	Ether·Ethanol (1:1)	37°
6	15 min	Ether	20°

(2) Cold TCA Insoluble Products

Treatment was similar to that shown in the above scheme with the substitution of a 15 min 5% TCA (4°) wash for Step 3.

F. RADIOISOTOPE COUNTING

A toluene-base scintillation phosphor, prepared by the addition of 4 g Omnifluor (New England Nuclear, Albany, N.Y.) per liter of scintillation-grade toluene, was used throughout for all radioassay experiments. Thoroughly dried samples on nitrocellulose or cellulose acetate membrane filters, or Whatman 3MM filter discs, were placed into thin-walled glass scintillation vials. Following the addition of 8 ml of scintillation fluid, the vials were capped tightly and counted in either a Beckman LS-200 B or Nuclear Chicago Model 720 liquid scintillation spectrometer.

When the radioactivity of samples containing dual isotopes was to be quantitated, the gain setting was adjusted to a value where the overflow between counting channels was restricted to the higher energy isotope. The amount of overflow of ^{14}C and ^{32}P radioactivity into the ^{3}H channel under these conditions generally was 35-40% and 5-10%, respectively.

A counting period of 5 min was normally adequate to give (99±1%) accuracy; longer periods sufficient to yield a 95±5% accuracy level, however, were used for samples with lower radioactivity. All sample values were corrected for background radioactivity registered in controls containing no added radioactivity.

G. DEPROTEINIZATION OF F-PILI CARBOHYDRATE MATERIAL

The aqueous phase from phenol-extracted F-pili material (procedure C (1), above) was found to contain a large amount of carbohydrate in association with residual traces of protein. To prepare this carbohydrate material for chromatographic analysis (see below), it was necessary to completely deproteinize this aqueous phase. This was accomplished by extraction with trichlorotrifluoroethane according to the procedure of Markowitz and Lange (1965).

1. One volume of aqueous, desalted carbohydrate material was mixed with one volume of trichlorotrifluoroethane and blended at high speed for 10 minutes with a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) in a 100 ml capacity blending cup which was packed in ice. This was to ensure complete and thorough homogenization. The casing was packed in ice because the boiling point of the solvent is 56°.

2. The resulting homogenate was then centrifuged (4°) at 5,000 x g for 10 minutes to separate the layers. Three layers were observed:

(a) an upper aqueous layer,

(b) a middle gel-like layer (containing protein), and

(c) a bottom layer of reagent.

The upper aqueous layer was removed by aspiration and re-extracted 2 times further with solvent following the same procedure.

3. This thrice "deproteinized" aqueous phase was then lyophilized to dryness and redissolved in 1.00 ml distilled water.

H. PAPER CHROMATOGRAPHY OF SUGARS

Chromatography of sugars associated with F-pili was performed by hydrolyzing the carbohydrate material in 0.48 N HNO_3 at 100° for 1 hour with subsequent separation of the sugars on Whatman No. 1 chromatography paper in 1-Butanol:Pyridine:Water (6:4:1) according to Putman (1957) and Noggle (1957). Sugars were visualized with either the diphenylamine-aniline-phosphoric acid spray (No. 8) or ammoniacal $AgNO_3$ spray (No. 234) of Krebs *et al*. (1969).

I. COLORIMETRIC ANALYSES

DNA and RNA were estimated colorimetrically using the orcinol and diphenylamine tests as described by Schneider (1957) using deoxyadenosine and adenosine as standards. Total phosphorus was estimated by the acid hydrolysis procedure of Fiske and SubbaRow (1925). Total protein was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as standard. Total carbohydrates were estimated as described in Chapter VI.

J. AMINO ACID ANALYSIS OF F-PILIN

Quantitative determination of the amino acid composition of protein hydrolysates was made on Beckman Model 120B and 121 automatic amino acid analyzers equipped with accelerated systems. About 20 nmoles of protein was hydrolyzed in 1.0 ml 6.7 N HCl for 24 hours at 110° in sealed evacuated tubes (18 mm x 151 mm). The hydrochloric acid was then removed *in vacuo* (5 to 50 μ) in a vacuum dessicator at room temperature (25°), and the residues analyzed in the amino acid analyzer. I am indebted to Mr. M. Nattriss for performing these analyses.

K. ELECTRON MICROSCOPY OF F-PILI

(1) Preparation of F-Pili for Electron Microscopy

A concentrate of F-pili was prepared by dissolving a small pH 4.0 F-pili pellet in 0.50 ml CTMM at 4°. A drop of the sample, or dilution thereof, was placed on a Formvar coated ion-charged 200 mesh nickel grid and the pili allowed to settle out for 1 hour at 4°. At this time, the grid was removed from beneath the drop, and residual moisture removed by touching the edge of the grid to a piece of bibulous paper.

Grids were then treated in one of three ways in order to identify the pili:

(a) negative staining with 2% phosphotungstic acid (Matheson, Coleman and Bell), pH 7.4, for one minute at room temperature by the drop method described above. The grid was washed one time in distilled water;

(b) positive-staining with 2% uranyl acetate (Mallinckrodt Chemical Works), pH 4.9, for 10 minutes at room temperature by the drop method described above. The grid was washed one time in distilled water; or

(c) shadowing with platinum-palladium at an angle of 12° with a back shadowing of carbon at an angle of 168°.

(2) Examination of F-pili

All samples were examined with a Philips EM-300 electron microscope (Philips Bedrijfsapparatuur n.v., Eindhoven, Holland) with an objective aperture of approximately 50 μm; a high tension accelerating voltage of 60 kV was used. An area on each grid was selected at random, and micrographs were taken with either Kodak electron-image plates or Kodak 35 mm electron-image film. Sample micrographs and descriptions are provided in Plates 3.1, 3.2 and 4.1.

I am indebted to Dr. Sterling Ainsworth, a postdoctoral fellow in this department 1969-1970, for performing the electron microscopy of crude and purified F-pili preparations.

CHAPTER III

THE QUANTITATIVE ASSAY OF F-PILI

To perform physical and chemical studies on isolated F-pili, three prerequisites must be satisfied:

(i) a method to assay relative amounts of F-pili in solution must be available;

(ii) milligram quantities of F-pili must be prepared; and

(iii) the F-pili must be available in a high state of purity.

This chapter summarizes the methods used and the results obtained in attempts to achieve the first of the foregoing criteria. The second and third conditions are dealt with in later chapters.

Estimation of F-pili levels in a bacterial culture or cell-free supernatant is currently accomplished by the bacteriophage adsorption assays of either Valentine and Strand (1965), Lodish and Zinder (1965), or Danziger and Paranchych (1970). All three assays rely on the fact that free male-specific RNA phage will pass through a membrane filter of pore size 0.45 to 0.65 μ, whereas phage-F-pili complexes remain on the filter pad. When radioactively labeled phage is used, the amount of F-pili trapped on the filter is measured indirectly by counting the radioactivity remaining with the filter after removal of free phage by washing.

The above assay techniques, even when used in conjunction with electron microscopy, provided only a qualitative estimate of F-pili content in a given sample (Valentine *et al.*, 1969), since no attempts had been made to exploit this method for the careful quantitation of F-pili. This chapter summarizes, therefore, modifications made to

the basic RNA phage adsorption assays to achieve nearly quantitative determinations of F-pili content. The assay is applicable to both whole cells in bacterial culture, and cell-free supernatants.

All measurement of F-pili recoveries have been expressed in terms of "cell-equivalents" of HB11 F^+ F-pili. E. coli HB11 were chosen as reference bacteria for the assay because they possess, on the average, one F-pilus per cell (Novotny et al., 1969a), and also because F-pili are the only appendages associated with this bacterium (Brinton and Beer, 1967). The term "specific activity", as used in relation to F-pili recoveries, is defined as the cell-equivalents of F-pili recovered in a given sample per µg of total protein (F-pilin) recovered in that same sample.

A. KINETICS OF PHAGE R17 ATTACHMENT TO F-PILI

The rationale for using the phage attachment procedure as a means of quantitating F-pili was based on the expectation that the addition of large amounts of phage to an F-pili preparation would result in the saturation of F-pili with attached phage. The radioactivity of the attached phage would then give a measure of the amount of F-pili in the system. To determine whether phage - F-pili interaction does, in fact, obey such simple saturation kinetics, the following experiments were performed.

(1) Time Course of Phage Attachment to F-Pili

A culture of E. coli F^+ HB11 bacteria was grown in lactose TMM culture medium at 37°, as described previously. At cell densities of 1.5×10^8, 3.0×10^8, and 5.0×10^8 bacteria/ml, 3 ml aliquots were removed from the culture, placed in separate reaction tubes, and

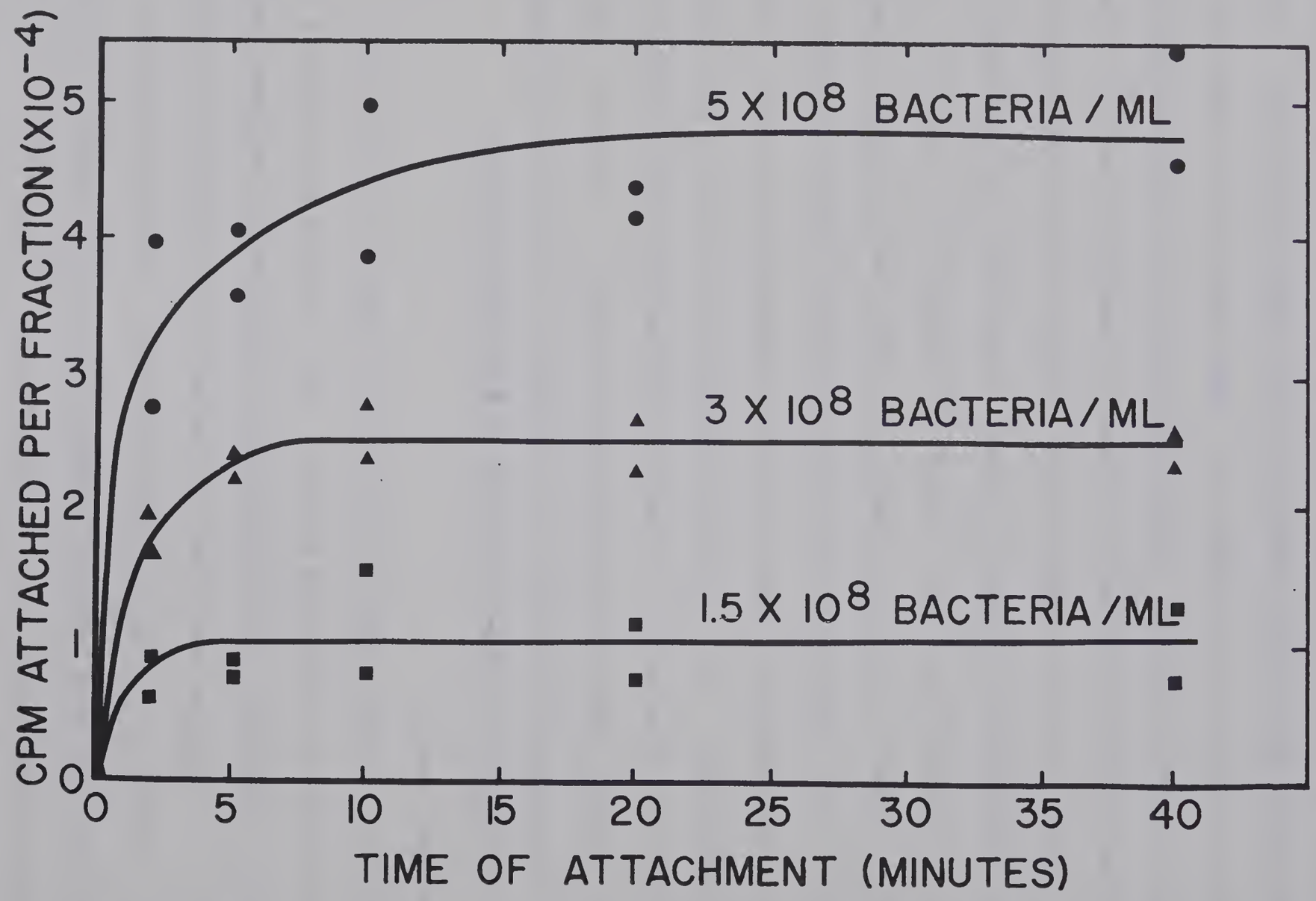
equilibrated to a temperature of 4° in an ice bath. Formaldehyde was then added to each culture to a final concentration of 0.40 M to fix the bacteria and prevent further outgrowth of F-pili. ^{32}P-labeled R17 phage was then added to each sample at an input ratio of 1000 phage particles per bacterium (see next section), and phage attachment was allowed to proceed. One ml samples were removed from the reaction mixtures at various time intervals and filtered through Gelman GA-6 triacetate filters (2.5 cm diameter; 0.45 μ pore size) which had been pre-soaked with lactose TMM. After washing the samples with three 5 ml portions of lactose TMM, the filters were dried and assayed for radioactivity in a liquid scintillation spectrometer.

An alternative procedure, used when Gelman GA-6 triacetate filters were in short supply, was to perform experiments in Z broth with filtration through Millipore nitrocellulose 0.45 μ membrane filters. Z broth was used with Millipore filters to suppress the otherwise very high levels of background radioactivity non-specifically trapped when TMM solutions containing ^{32}P-labeled R17 phage were passed through these nitrocellulose membranes (see Brinton and Beer, 1967; Danziger and Paranchych, 1970, for further details).

All filtration was accomplished under a vacuum of 6 inches of mercury, using a multiple-head filter box with 5 sets of Millipore spring-clamped sintered-glass filters. Determination of radioactivity trapped on the membrane filters is described in Chapter II. The assay was performed at 4°, since phage - F-pili complexes form readily at this temperature, but penetration of phage RNA into the cell does not occur (Ippen, 1967). Thus, only adsorption was measured.

The results of this experiment are given in Figure 3.1. It was

Figure 3.1. Kinetic profiles, at 4°, of the adsorption of ^{32}P-labeled R17 phage, at an input ratio of 1000 phage particles per cell, to various cell densities of HB11 F^+ bacteria grown in complete lactose TMM.


CPM ATTACHED PER FRACTION (X10⁻⁴)
0
1
2
3
4
5
0
5
10
15
20
25
30
35
40
TIME OF ATTACHMENT (MINUTES)
5 X 10⁸ BACTERIA / ML
3 X 10⁸ BACTERIA / ML
1.5 X 10⁸ BACTERIA / ML

found that the time required for maximum adsorption of R17 phage to F-pili to occur varied directly with the cell concentration, 5 minutes being required for a bacterial culture at 1.5×10^8 cells/ml, 10 minutes for a culture at 3×10^8 cells/ml, and so on. Although not shown in Figure 3.1, studies were often carried out on cell densities as high as 8×10^8 bacteria/ml. At this bacterial concentration, maximum adsorption was achieved by about 25-30 minutes. Thus, a period of 40 minutes was chosen as a standard time for adsorption of phage to F-pili in all subsequent assays.

(2) The Effect of Phage Concentration on Phage Attachment

The following experiment was performed to determine the saturating amount of R17 phage per cell-equivalent of F-pili. A culture of HB11 F^+ bacteria was grown at 37° in complete lactose TMM to mid log-phase (4×10^8 bacteria/ml). The culture was chilled to 4° in an ice bath, after which formaldehyde was added to a final concentration of 0.40 M. Two-ml aliquots were removed from the culture flask and placed in separate reaction tubes, to which ^{32}P-labeled R17 phage was then added at multiplicities varying from 100 to 6000 phage particles per bacterium. The phage particles were allowed to attach to the F-pili for 40 minutes at 4°, at which time 1 ml samples of the reaction mixture were removed and filtered through GA-6 membrane filters, as previously described. The number of phage particles attached per F-pilus was determined from the specific activity of the phage (i.e., phage particles per cpm), and these values were plotted against the input phage concentration. This curve is shown in Figure 3.2(a). As can be seen, saturation of the F-pilus by phage starts at an input of approximately 1000 phage particles per bacterium, and is essentially complete at an input of 6000 phage particles

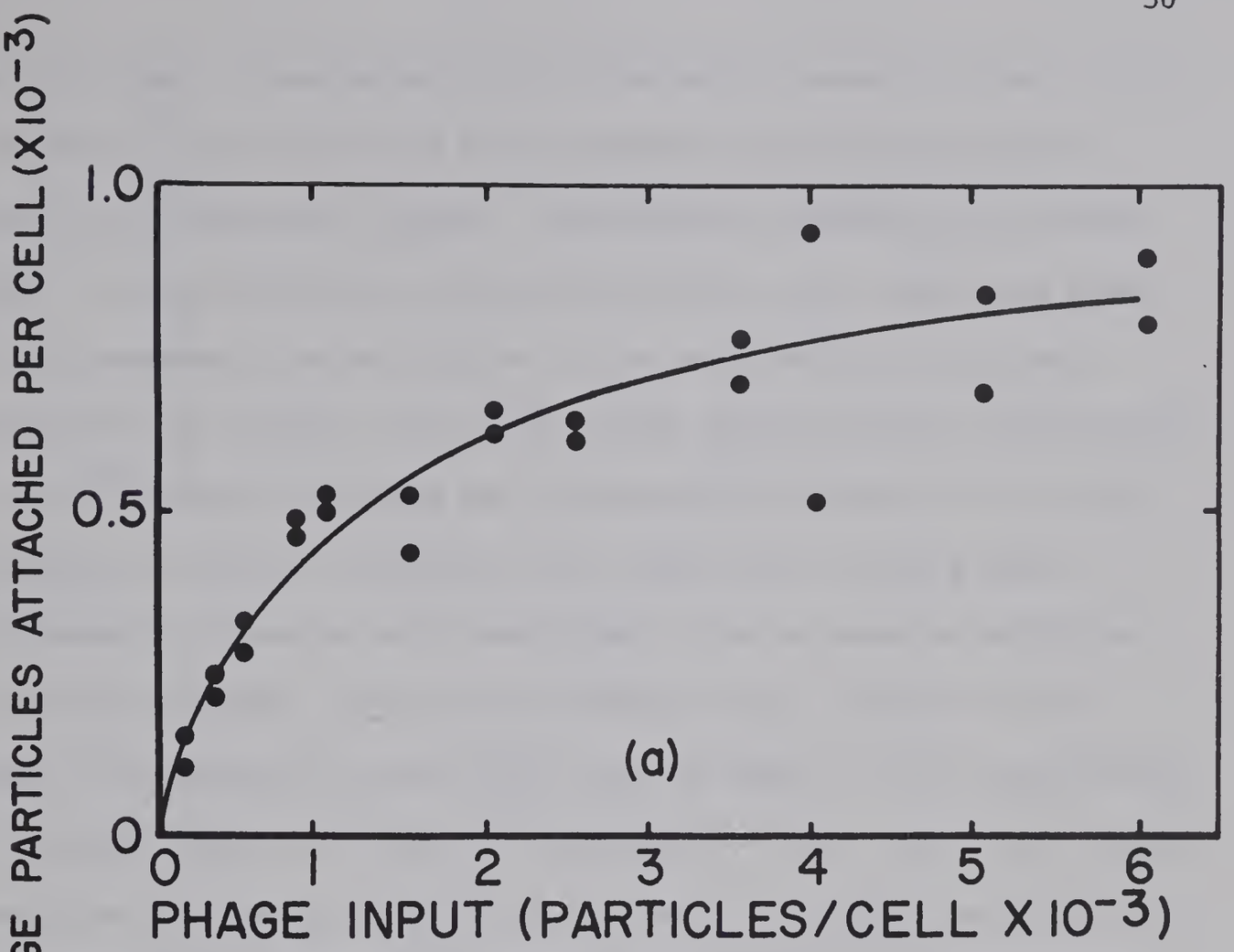
Figure 3.2. (a) The kinetic attachment-profile of various input multiplicities of ^{32}P-labeled R17 phage to 4×10^8 HB11 F^+ bacteria in complete lactose TMM at 4°.

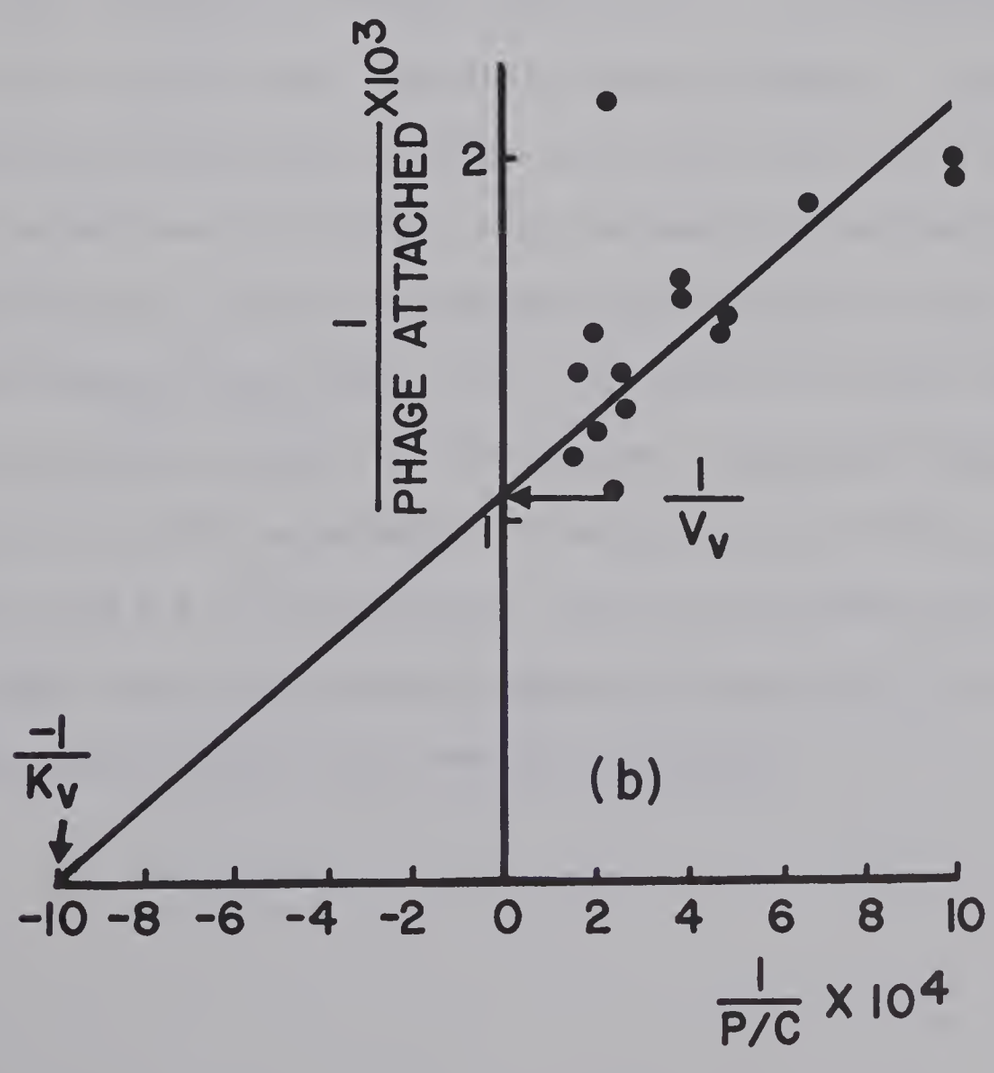
(b) Reciprocal plot of data shown in Figure 3.2(a).

$K_v = 1000$. This represents the p/c ratio of input phage that will produce saturation of 1/2 of the total available phage receptor sites per F-pilus.

$V_v = 970$. This represents the maximum number of phage receptor sites saturated by an infinite amount of input phage after 40 minutes of attachment to F-pili at 4°.

Curve (b) fitted using the least-squares principle.


PHAGE PARTICLES ATTACHED PER CELL (X10⁻³)
1.0
0.5
0
0
1
2
3
4
5
6
PHAGE INPUT (PARTICLES/CELL X 10⁻³)
(a)


1/PHAGE ATTACHED X10³
2
1
1/Vv
1/Kv
(b)
-10 -8 -6 -4 -2 0 2 4 6 8 10
1/P/C X 10⁴

per bacterium. A reciprocal plot of the data is shown in Figure 3.2(b), from which it was calculated that a maximum of 970 phage adsorption sites are available per F-pilus. Although 970 represents an average number of phage adsorption sites per bacterium, this number was found to vary somewhat from one culture to the next, and with one phage preparation to the next. For this reason, and because the radioactivity of the ^{32}P-labeled R17 phage was continuously decaying, it was found essential to prepare a standard curve (see below) relating phage attachment to bacterial cell density each time an assay of cell-free F-pili was performed. The value of 1000 p/c (K_v) (Figure 3.2) was chosen as a minimal but sufficient input of phage to allow quantitation of relative numbers of F-pili in suspension, as zero order phage attachment kinetics were approached when this amount of phage was used. Although this value represents one-half saturation of a cell-equivalent of F-pili, it was, nonetheless, adequate for assay purposes. Standard curves of counts per minute R17 attached, at an input of 8×10^{11} phage particles per ml of reaction mixture, with increasing concentrations of HB11 bacteria yielded a linear relationship over the range of cell concentrations examined (see Figure 3.3). The value of 8×10^{11} phage particles/ml represents an input of 1000 p/c for a bacterial culture at a cell density of 8×10^{8} bacteria/ml. It should be noted that at cell densities less than 8×10^{8} bacteria/ml, the p/c ratio becomes substantially higher than 1000, providing adequate saturation of the viral receptor sites per F-pilus at the lower cell densities.

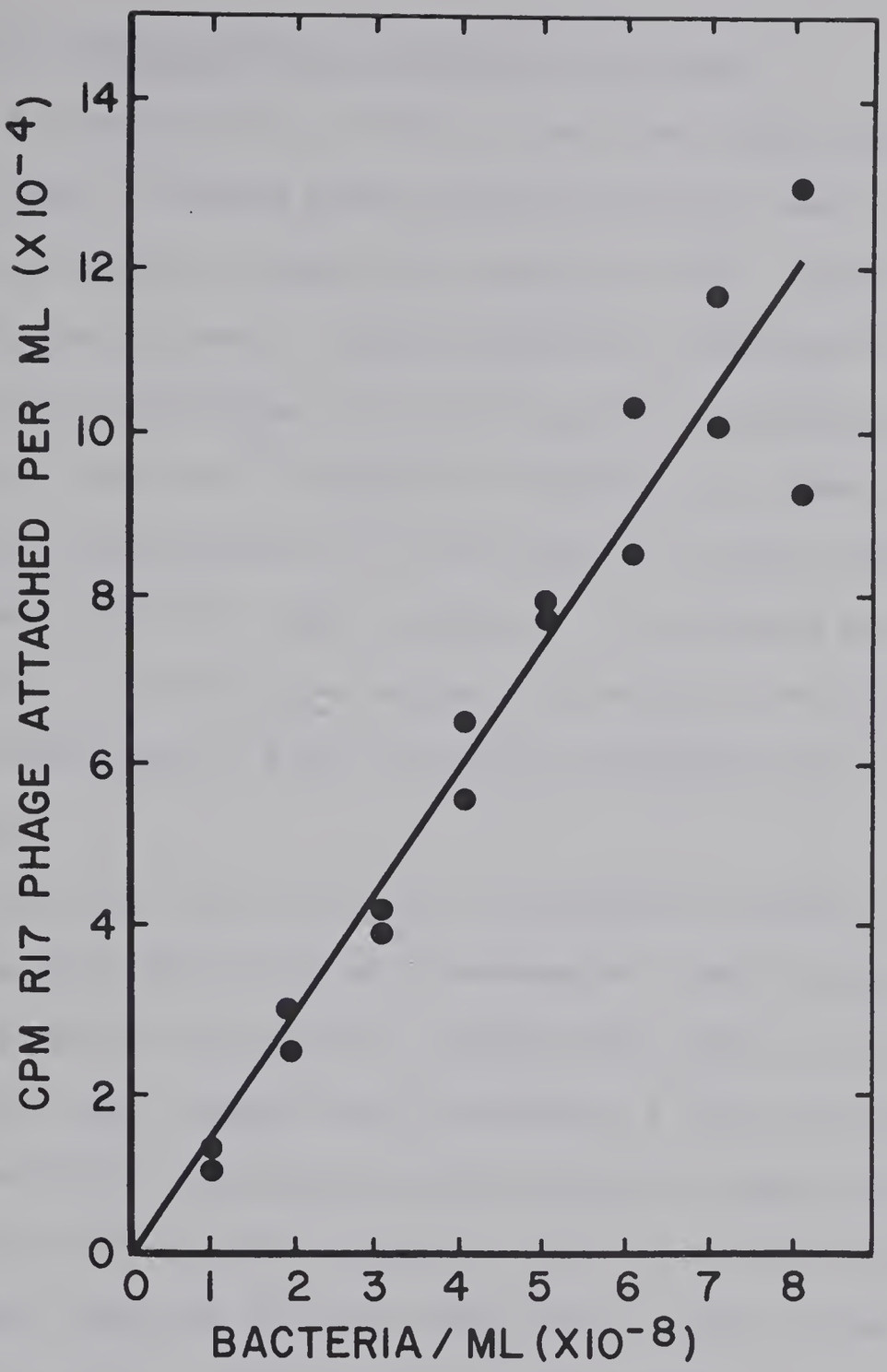


Figure 3.3. A standard curve of cpm R17 phage attached, at an input of 8×10^{11} phage particles/ml, to increasing concentrations of HB11 bacteria. The value of 8×10^{11} phage particles/ml represents an input of 1000 p/c for a bacterial culture at 8×10^{8} cells/ml. Curve fitted using the least-squares principle.

B. THE DETERMINATION OF CELL-FREE F-PILI LEVELS

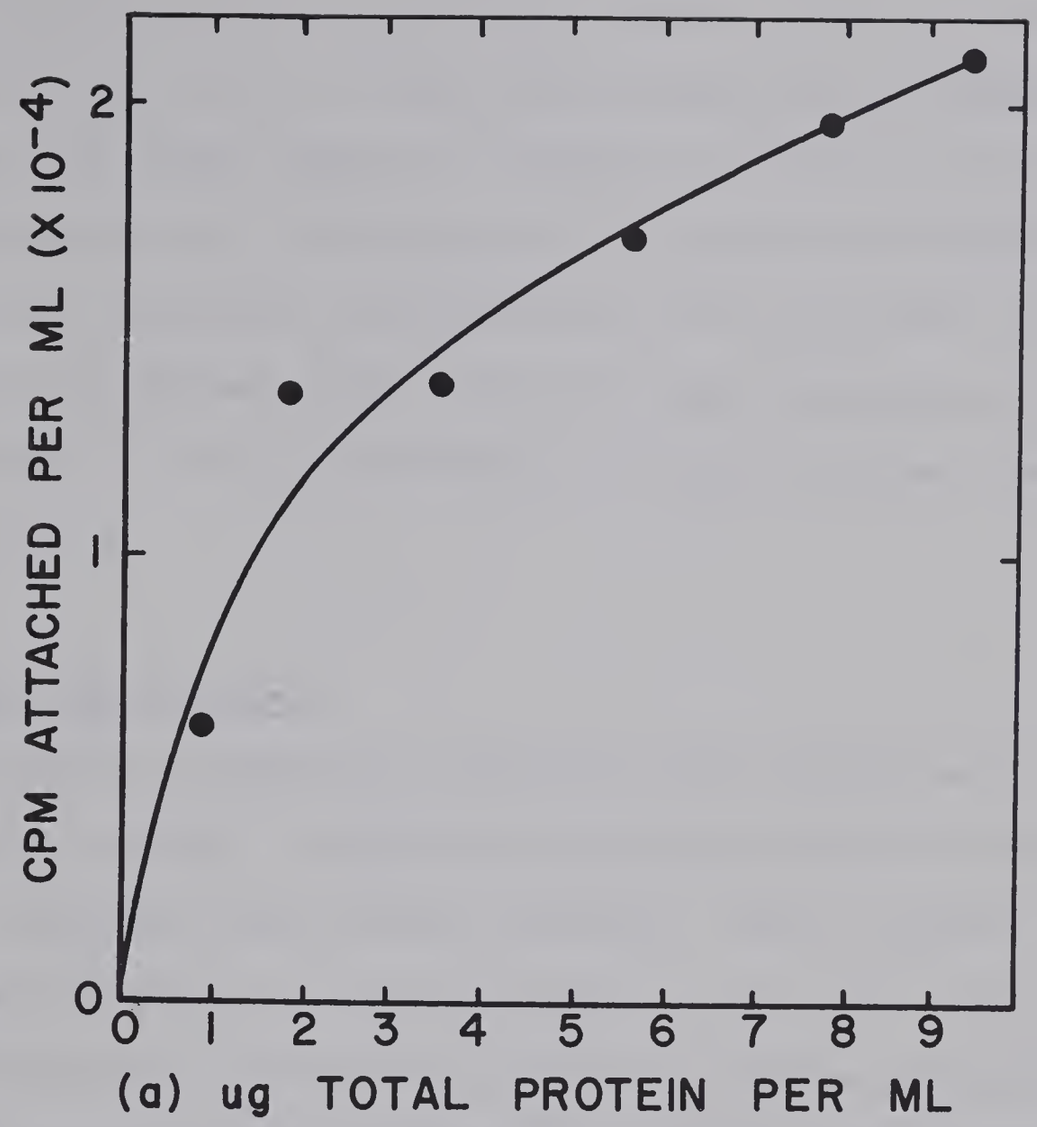
The quantitation of F-pili in cell-free preparations was accomplished as follows. Standard curves relating counts per minute ^{32}P-labeled R17 phage attached to varying cell densities of HB11 bacteria (described above) were prepared. Reaction mixtures, containing serial dilutions of F-pili concentrates (0.1 to 1.0 µg total protein/ml; see below) and 8×10^{11} particles ^{32}P-labeled R17 phage/ml, were made in lactose TMM medium, and incubated at 4° for 40 minutes to allow complete attachment of phage to F-pili. After incubation, 1 ml aliquots were removed and filtered. The counts per minute attached per ml were converted into cell-equivalents of F-pili by direct extrapolation from the standard curve.

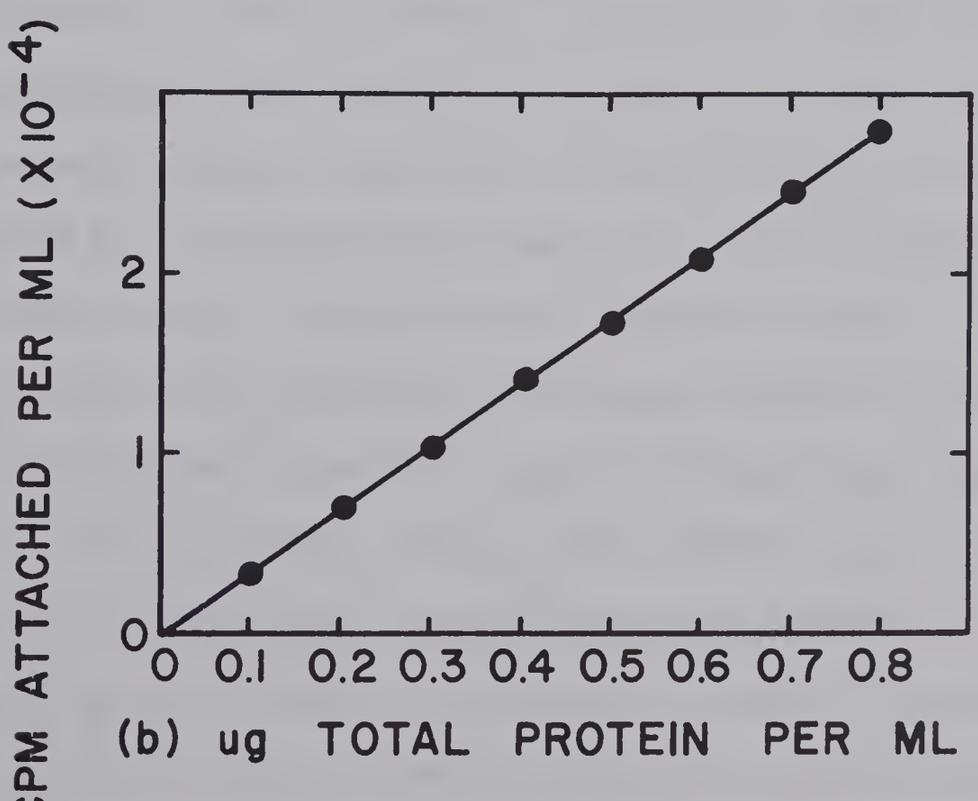
Purified, cell-free F-pili concentrates contained, on the average, 170 µg total protein per ml of suspension. The following experiment was performed to determine a concentration range of F-pilin which would produce, under standard assay conditions, a linear increase relationship between F-pili concentration and the amount of phage attachment. Various dilutions of an F-pili concentrate (172.3 µg total protein/ml of suspension) were made in lactose TMM so that between 1.0 and 10.0 µg F-pilin per ml of reaction mixture were assayed. Two-ml reaction mixtures were prepared. 1.6×10^{12} particles R17 phage were added to each tube, giving a final concentration of 8×10^{11} virus particles/ml. The phage was allowed to attach for 40 minutes at 4°, after which time 1 ml aliquots were removed from the reaction tubes, filtered as previously described, and assayed for radioactivity. Results obtained by this procedure are given in Figure 3.4(a). It can be seen that whereas phage attachment appears linear at low levels of F-pili, it is non-linear

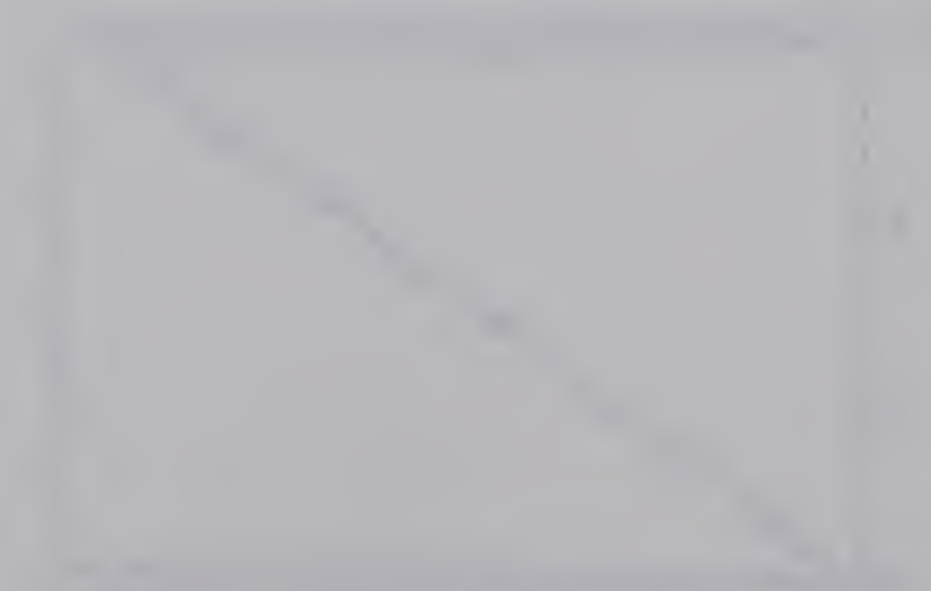
Figure 3.4. (a) The adsorption, at 4°, of ^{32}P-labeled R17 phage to various concentrations (1 to 10 µg total protein) of a purified cell-free F-pili concentrate in lactose TMM. Experimental details are described in the text.

(b) The adsorption, at 4°, of ^{32}P-labeled R17 phage to various concentrations (0.1 to 0.8 µg total protein) of a purified cell-free F-pili concentrate in lactose TMM. Experimental details are described in the text.

Phage preparations of different specific activities were used for the assays shown in (a) and (b).


CPM ATTACHED PER ML (X 10⁻⁴)
0
1
2
0 1 2 3 4 5 6 7 8 9
(a) ug TOTAL PROTEIN PER ML


CPM ATTACHED PER ML (X 10⁻⁴)
0
1
2
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
(b) ug TOTAL PROTEIN PER ML



at concentrations above 1.0 µg of F-pili protein. This is not entirely surprising, since F-pili, at high concentrations, tend to aggregate into rope-like bundles (Plate 3.1) leading to the loss of many potential phage adsorption sites. When the F-pili preparation was diluted to a concentration less than 1.0 µg of protein, on the other hand, F-pili aggregation was minimal (Plate 3.2), and a linear response was obtained, in the range 0 to 0.8 µg of protein/ml, for phage attachment (see Figure 3.4(b)).

C. SUMMARY AND DISCUSSION

The results of experiments presented in this chapter may be summarized as follows. A method for quantitating F-pili in suspension by phage adsorption assay has been developed. Both an adequate time for maximal R17 phage attachment to F-pili to occur, and a saturating amount of phage per cell-equivalent of F-pili have been determined. Standard curves of counts per minute ^{32}P-labeled R17 phage attached to bacteria *vs*. varying cell density (cell-equivalents F-pili) were prepared, from which unknown quantities of cell-free F-pili in suspension were measured by extrapolating the number of c.p.m. ^{32}P-labeled R17 phage attached per µg of unknown material (total protein).

The rationale for establishing the phage attachment assay on a quantitative basis was to provide a means of determining, stoichiometrically, F-pili recoveries during their isolation from whole cells, and subsequent purification. Although the assay is capable of determining the relative amount of F-pili in suspension in terms of cell-equivalents per unit volume, it does not provide information regarding the actual *length* of F-pili being assayed. Moreover, the assay provides only

Plate 3.1. Electron micrograph of F-pili.

Concentrated F-pili, which had been purified by the procedure outlined in Chapter V, were stained with 2% phosphotungstic acid for one minute and examined under a Philips EM 300 electron microscope. Magnification = X 40,000.

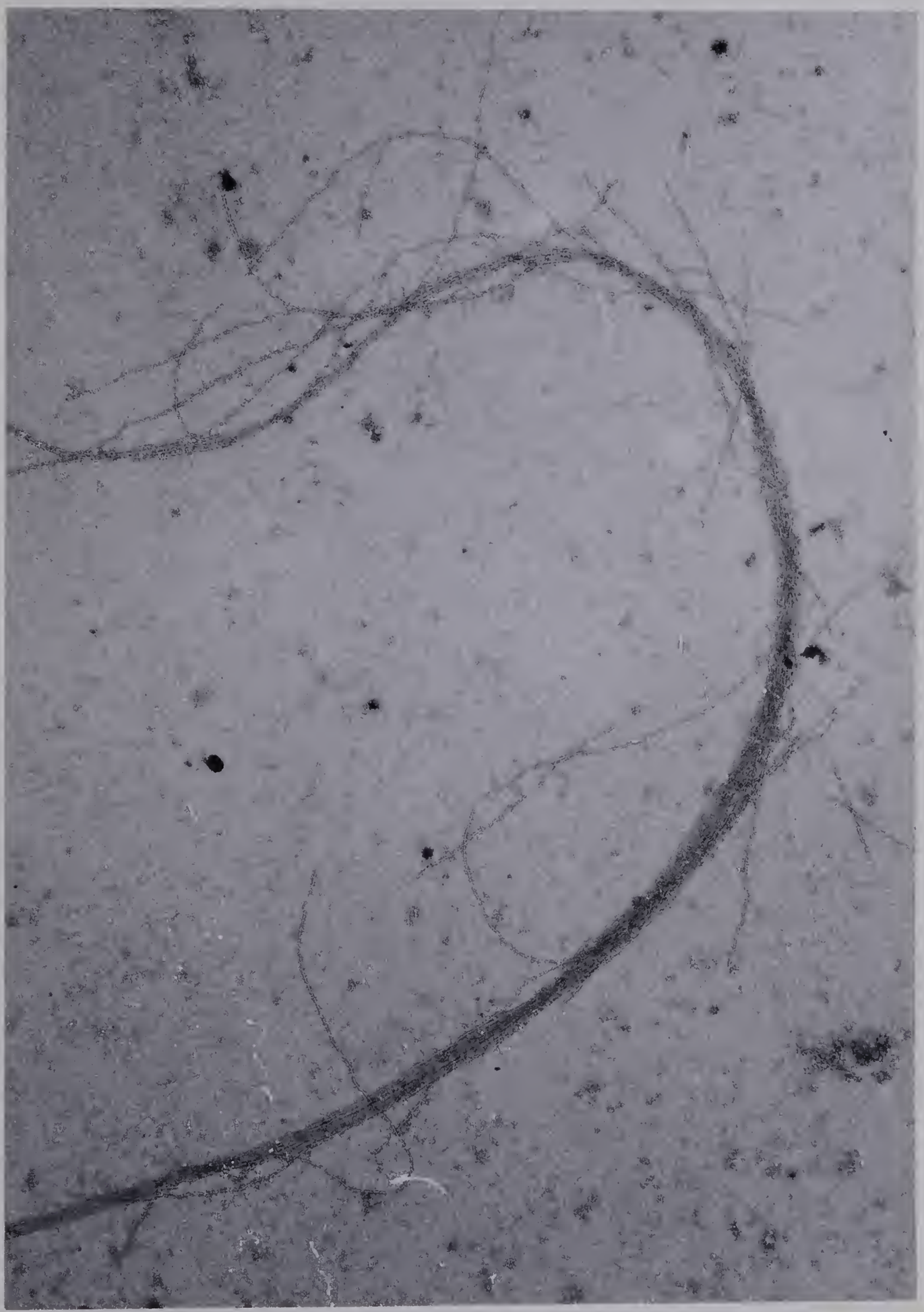
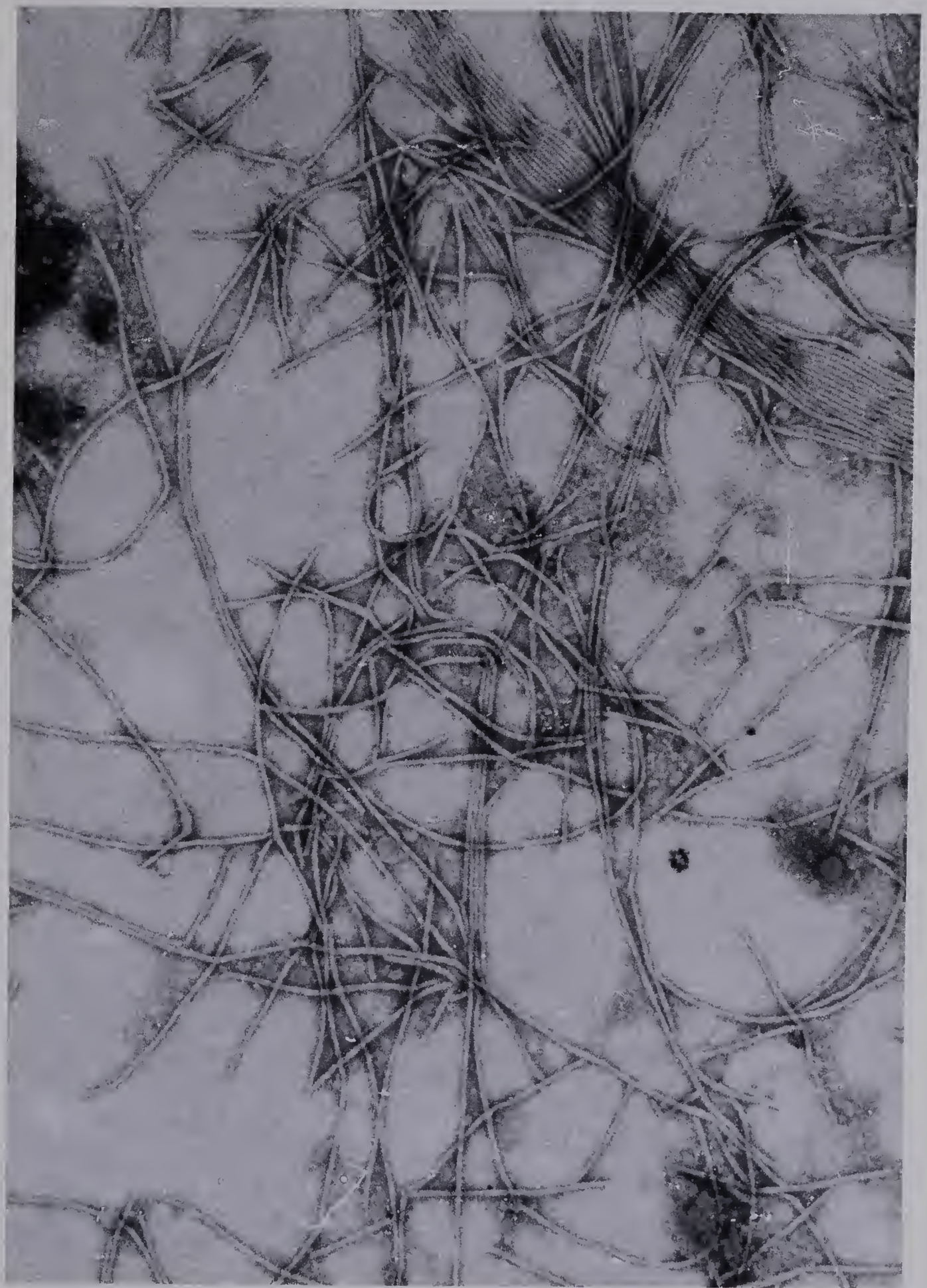


Plate 3.2. Electron micrograph of F-pili.

"Purified" F-pili, diluted to a concentration of 1.0 µg total protein/ml, were stained with 2% phosphotungstic acid for one minute and examined under a Philips EM 300 electron microscope. Magnification = X 160,000.



limited information regarding the state of aggregation of the F-pili being assayed. Although dilution effectively eliminates the "bundling" of F-pili into rope-like threads, it is possible that any combination of end-to-end or side-to-side aggregation of single F-pili strands may occur, as shown in Plate 3.2, the electron micrograph of a 200 fold dilution of an F-pili concentrate.

Despite these shortcomings, however, the assay allowed F-pili preparations, at various stages of purification, to be monitored quickly and easily for F-pili content. When additional information regarding the physical state of the F-pili (i.e., length and aggregation) was required, the samples were further examined in the electron microscope.

CHAPTER IV

THE ISOLATION OF F-PILI FROM BACTERIA

Having developed a procedure for the quantitative assay of F-pili, further studies were carried out to find a method by which F-pili could be obtained in high yield from bacterial cultures and then purified from contaminant cellular material. This chapter deals with a comparison of various methods used to isolate F-pili from host bacteria, and the selection of the best of these procedures for use in the large-scale preparation of cell-free F-pili.

To date, F-pili removal from bacteria has commonly been achieved either by shearing whole cultures in a mechanical blendor, or simply by collecting F-pili which have become spontaneously separated from cells in the culture medium—i.e., by natural outgrowth or shear forces created normally in the culture by the aeration process. Preliminary attempts to exploit either of these methods for removal of F-pili from bacteria proved somewhat less than satisfactory, in that very low yields of cell-free F-pili were recovered. It was thus realized that a careful study of these depiliation procedures would have to be undertaken to determine the most efficient means of removing F-pili from the host cells. This study is described below.

A. SPONTANEOUS RELEASE OF F-PILI IN NORMALLY GROWING CULTURES OF E. COLI

Brinton and Beer (1967), Ippen (1967) and Valentine *et al*. (1969) have shown that natural release of F-pili from bacteria in normally-growing cultures increases exponentially during the log-phase stage of growth, reaching a maximal value of one-half of the total F-pili

population at a cell density between 5 x 10^8 and 7 x 10^8 bacteria/ml. Cultures of cell density greater than 7 x 10^8 bacteria/ml were shown (Brinton and Beer, 1967) to produce no further increase in total or cell-free F-pili levels, a direct indication of ensuing stationary-phase growth in most F-piliated bacterial strains (Valentine et al., 1969). From the data provided in the above articles, it was, unfortunately, not possible to determine whether this spontaneous loss of F-pili by the growing cells was due to continuous outgrowth and "dropping off" of F-pili, or to shear forces created by the method of aeration used by these workers. In our studies, the method of aeration was designed to minimize turbulence in the culture, the method used being that of slow shaking (125 revolutions per minute) in a rotary shaking water bath calibrated to a temperature of 37°. It was reasoned that any level of free F-pili formed under these conditions would be due to a truly spontaneous release of F-pili by the cell.

An experiment was thus carried out to determine if F-pili could be recovered in significant amounts from cultures of HB11 bacteria without the aid of mechanical shearing procedures. The percent natural release of F-pili was determined in cultures varying in density from 2.5 x 10^8 bacteria/ml (early log-phase) to 2 x 10^9 bacteria/ml (stationary phase). Fifty-ml cultures of HB11 bacteria were grown in complete lactose TMM as described in Chapter II. At the desired cell densities, 10 ml aliquots were removed from the parent culture, formaldehyde added to each to 0.40 M, and the temperature of each adjusted to 4° in an ice bath. All samples resulting from aliquots of cell density greater than 2.5 x 10^8 bacteria/ml were then diluted to 2.5 x 10^8 bacteria/ml with fresh medium containing 0.40 M formaldehyde. Each aliquot was divided

in half, one portion serving as an untreated control, the other undergoing differential centrifugation at 8000 x g for 10 minutes to remove the bacterial cells. The supernatants from the centrifuged fractions were removed, the cell pellets resuspended to their original density in fresh lactose TMM containing 0.40 M formaldehyde, and all fractions then assayed for F-pili. Results of this experiment are given in Figure 4.1.

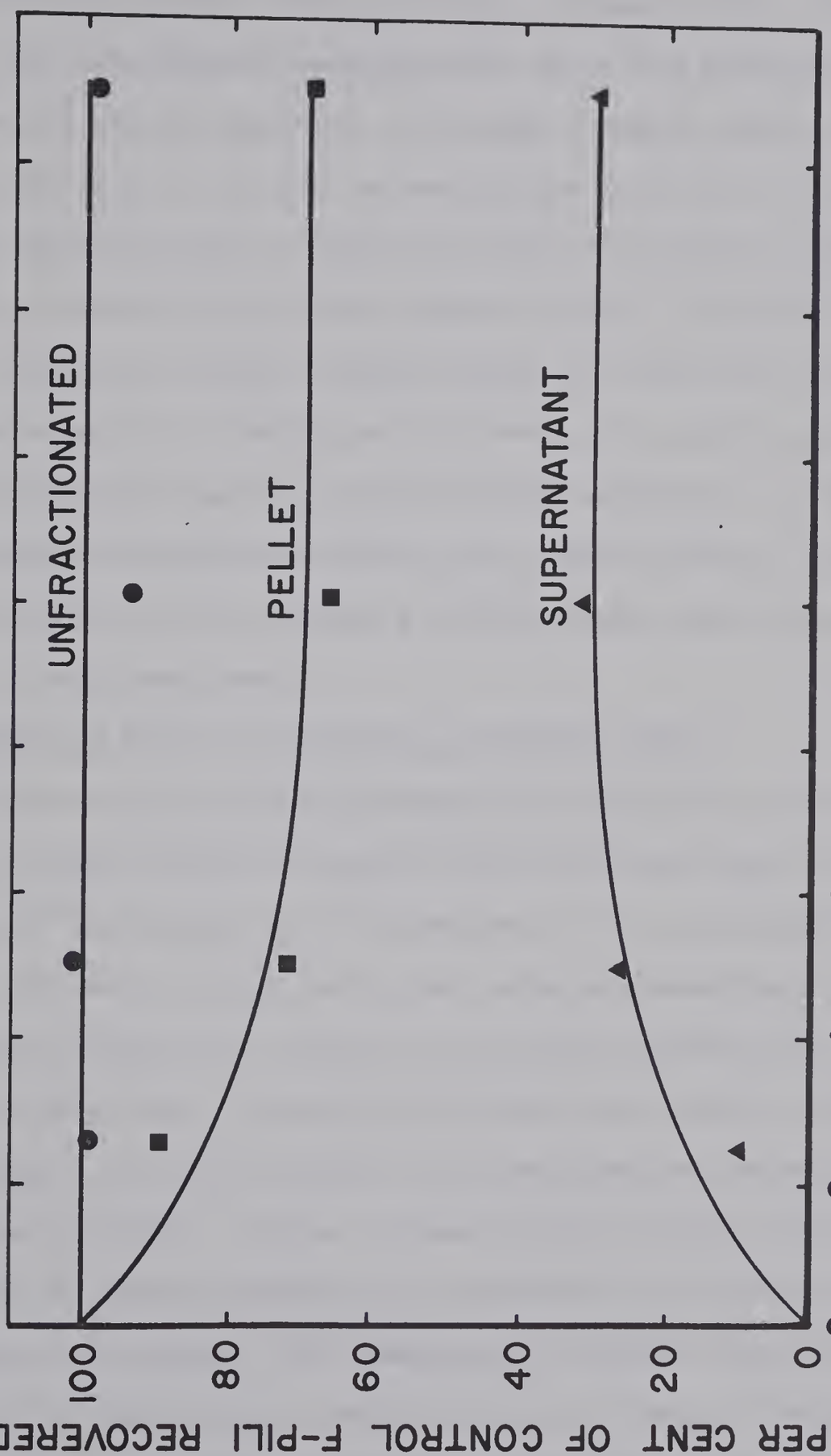
Normally-grown cultures of HBl1 bacteria were found to produce a maximal level of 30% naturally-released F-pili in culture supernatants at a cell density of 7×10^8 bacteria/ml. The practical significance of this fact is that 30% of all F-pili in HBl1 bacterial cultures can be gathered from suspension just by allowing cell replication to proceed to 7×10^8 bacteria/ml, fixing and harvesting the cells by formaldehyde treatment and differential centrifugation, and using the supernatant phase as the starting point in a purification procedure. Valentine et al. (1969) have reported that a culture of HB45 bacteria, grown normally in tryptone yeast-extract medium at 37° to a cell density of 5×10^8 bacteria/ml, extrudes, by natural-release, some 50% of the total F-pili population into the supernatant phase. It is possible that the presently reported, and lower, value of 30% natural-release of F-pili into the supernatant phase of normally grown HBl1 cultures is due to the fact that a less nutritious and completely synthetic growth medium was used for these experiments.

B. MECHANICAL REMOVAL OF F-PILI BY BLENDING OF BACTERIAL CULTURES

The technique of removing F-pili from male strains of bacteria by blending of whole cultures was originally developed and first introduced

Figure 4.1. The per cent natural release of F-pili in normally grown lactose TMM cultures of *E. coli* HB11 bacteria.

Bacterial cultures were grown at 37° with rotary water bath shaking (125 revolutions per minute) to the cell densities indicated. After fixing and diluting the bacteria (see text for details), cells were removed from the suspension, and F-pili levels quantitated in both supernatant and bacterial pellet fractions. Values plotted represent the average of at least 5 measurements.


PER CENT OF CONTROL F-PILI RECOVERED
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
BACTERIA PER ML (X 108)
UNFRACTIONATED
PELLET
SUPERNATANT

by Brinton in 1964-65, who, at the time, was studying the genetic origin of F-pili and the chemistry of Type I (common) pili. The method used at that time employed blending speeds which were sufficiently high to remove all surface appendages, and possibly unknown surface substances that could not be seen in the electron microscope. To quantitatively relate the loss of F-pili and F-pili function(s) to the removal of these structures from the cell surface, Novotny, Carnahan and Brinton (1969a) developed a precise blending technique by which one could determine the intensity of blending and the removal of a surface structure (the blending spectrum) for any given surface appendage. As this refined blending technique was suited to the identification of a blending spectrum for HB11 F-pili, Novotny's techniques were used as a guideline in the following experiments.

(1) Removal of F-pili as a Function of Blending Speed

To determine the minimum blending speed required for removal of F-pili from HB11 bacteria, cultures of HB11 cells were grown to a density of 5.5×10^8 bacteria/ml in Z broth medium at 37°, as described in Chapter II. The use of Z broth in this and subsequent experiments, as explained in Chapter III, was due to a shortage of Gelman GA-6 triacetate filters at this time. On reaching the desired cell density, the cultures were chilled to 4° in an ice bath, and formaldehyde was added to a concentration of 0.40 M. Portions of these cultures (40 ml) were placed into a 200 ml capacity blending cup, and blended at one speed in a Sorvall Omni-Mixer for 2 minutes. The temperature of aliquots being blended was kept at 4° by immersing the blender cup in an ice bath. Blending speeds, in terms of r.p.m., were measured with a Jaquet Precision Tachometer (Herman H. Sticht Co., Inc., New York, N.Y.). The bacterial cells were

removed from the blended specimens by differential centrifugation at 8000 x g for 10 minutes, and the supernatant phases removed. After resuspending the pelleted bacteria in fresh Z broth (containing 0.40 M formaldehyde) to their original cell density, the suspension was assayed for F-pili content. Results of this procedure are shown in Figure 4.2.

The curve presented in Figure 4.2 describes the blending spectrum, at 4°, for HBl1 F-pili in Z broth containing 0.40 M formaldehyde. Results show that no significant loss of cell-associated F-pili occurred when the cells were blended at speeds less than 2000 revolutions per minute. Blending speeds in excess of 2000 r.p.m., however, produced a sharp and continuous decrease in the number of cells possessing F-pili. The efficiency of blending was not affected by the type of growth medium employed, as an identical curve was later obtained for HBl1 bacteria grown in lactose TMM (unpublished results). It was consistently observed that blending the enriched medium at high speeds produced a considerable amount of foaming. Treatment of broth cultures with a silicone-type antifoam, post-blending, eliminated this condition. Blending speeds in excess of 3000 r.p.m. were shown, by electron microscopy, to produce substantial fragmentation of F-pili (see Plate 4.1). This observation was expected, as a combination of shear forces which simultaneously tear F-pili from the cell wall and break these structures into small pieces is unavoidable in this procedure (Novotny et al., 1969a). To minimize the amount of F-pili breakage during blending, a blending speed just sufficient to remove these filaments from the cell wall was incorporated into all blending procedures. A blending speed of 2500 r.p.m. was selected from the profile in Figure 4.2 to best fulfill this condition.

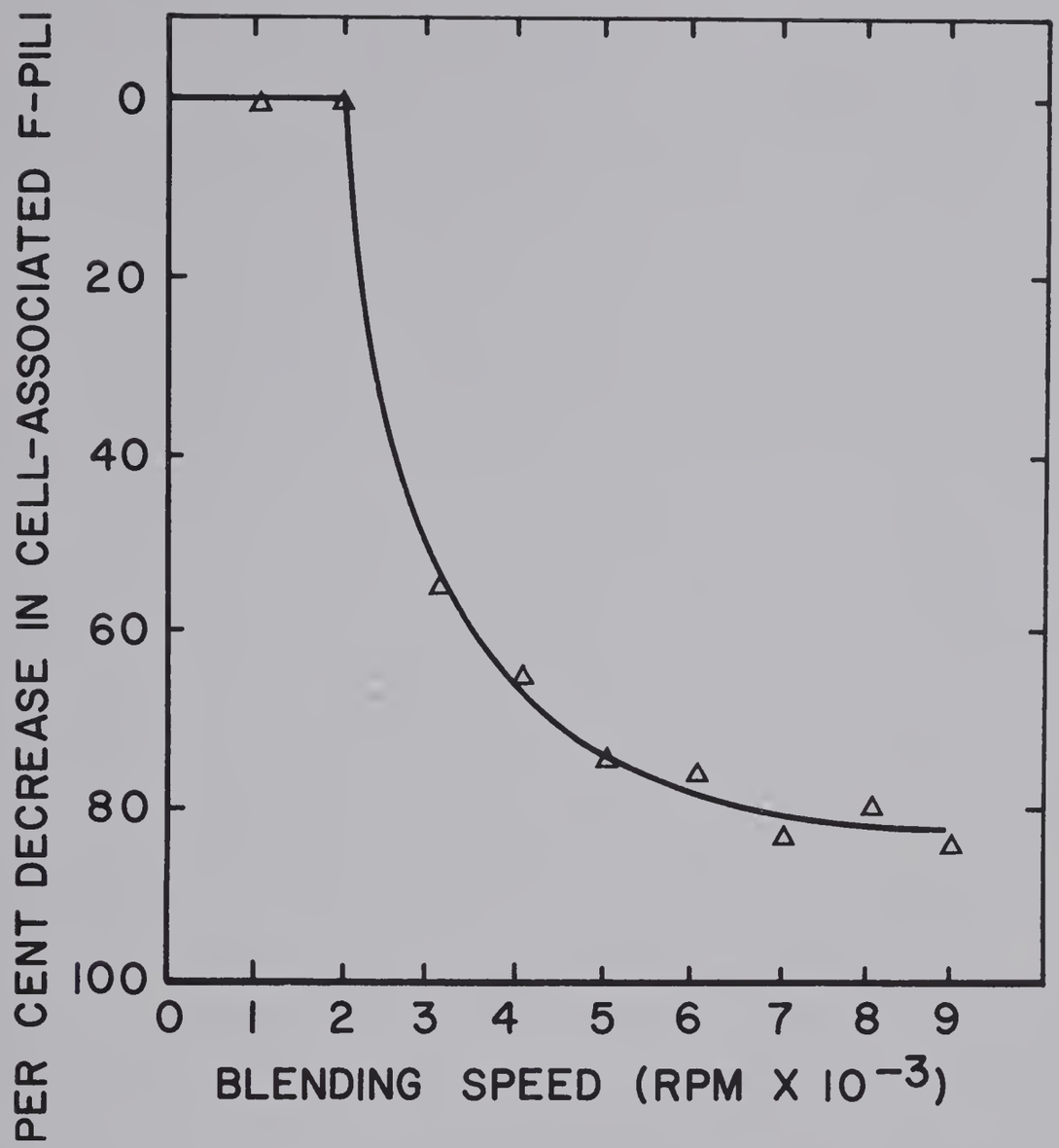
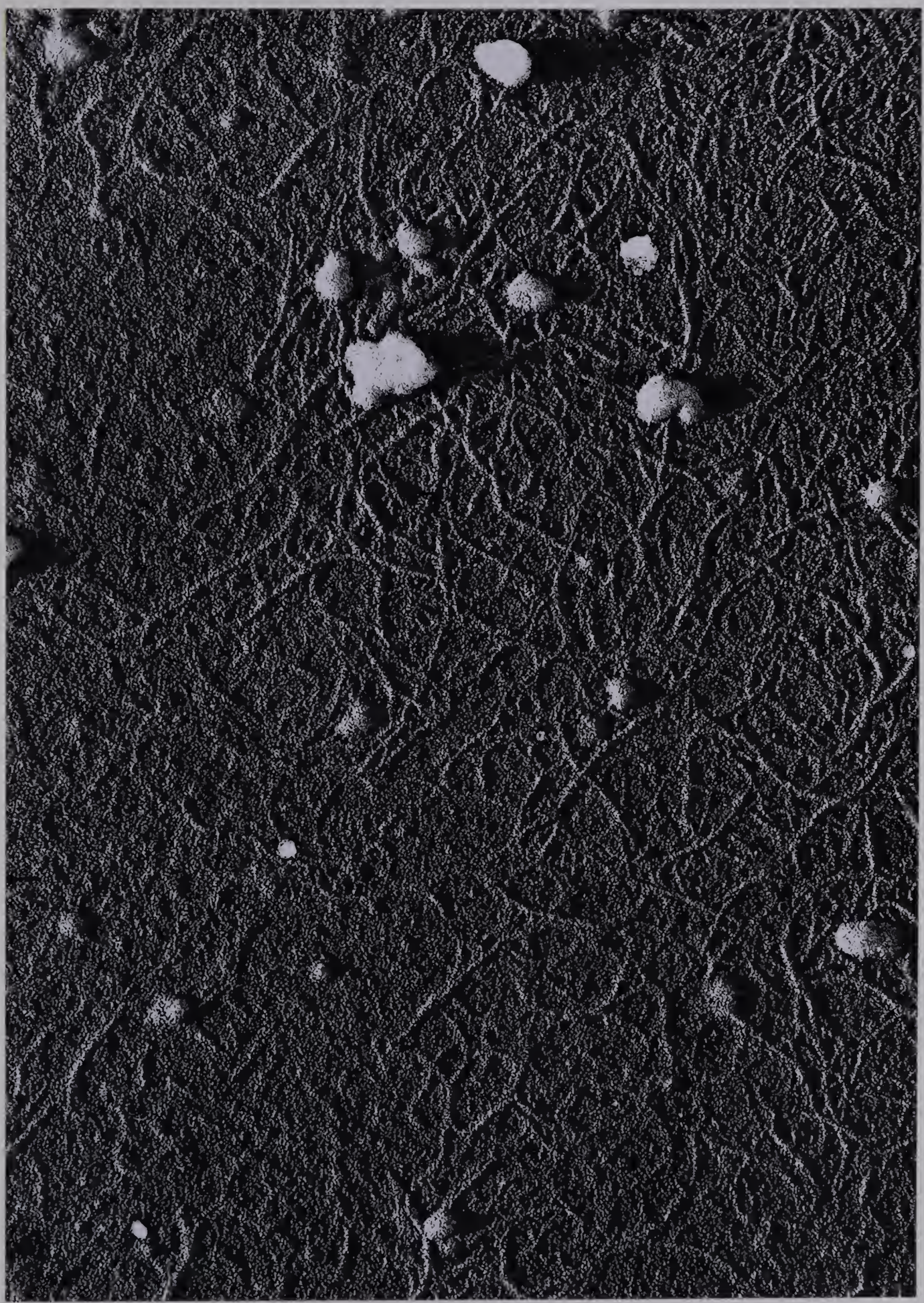


Figure 4.2. Decrease in cell-associated F-pili as a function of blending speed.

HB11 cells were blended for 2 minutes at the indicated speed. Cultures were blended at 4° in Z broth medium containing 0.40 M formaldehyde. Values plotted represent the average of at least 5 measurements. All values represent cell-associated F-pili only.

Plate 4.1. Electron micrograph of fragmented F-pili.

Purified F-pili, diluted to a concentration of 1.0 µg total protein/ml, were blended at 3000 revolutions per minute for 30 seconds in a Sorvall Omni-Mixer (see text) and processed for electron microscopy. Grids, onto which F-pili fragments were allowed to settle for 5 hours, were overshadowed with Pt-Pd, and examined in a Philips EM 300 electron microscope. The globular-like material was not identified. Magnification = X 240,000.



(2) Removal of F-Pili as a Function of Time of Blending

To determine a minimal time of blending required for the removal of F-pili from HB11 bacteria, the following experiment was performed. HB11 bacteria were grown in Z broth medium at 37° to a cell density of 5.5×10^8 bacteria/ml, as described previously. On reaching the desired cell density, the cultures were chilled to 4° in an ice bath, and formaldehyde added to a concentration of 0.40 M. Portions of these cultures (40 ml) were placed into a 200 ml capacity blending cup, and blended at a speed of 2500 r.p.m. in a Sorvall Omni-Mixer for various time intervals ranging from 0.5 to 2.0 minutes. The temperature of aliquots being blended was kept at 4° by immersing the blendor cup in an ice bath. The bacterial cells were removed from the blended specimens by differential centrifugation at 8000 x g for 10 minutes, and the supernatant phases removed. After resuspending the pelleted bacteria in fresh Z broth containing 0.40 M formaldehyde to their original cell density, the suspension was assayed for F-pili content. Results of this procedure are given in Figure 4.3.

The curve presented in Figure 4.3 describes the removal of F-pili from HB11 bacteria as a function of time of blending. Results show that after blending of the parent culture at 2500 r.p.m. for 0.5 minute, 40% of the cell-associated F-pili fraction was converted into the cell-free state. This increased to a maximum of 45% after 2.0 minutes of blending, correlating well with the value of 46% found from extrapolation in Figure 4.2. As 25% of the total F-pili population is present in the cell-free state from natural release at the cell density of culture used (see Figure 4.1), the true level of cell-free F-pili present after blended ranged from 65 to 70% of the control. As blending for time periods between 0.5 and 2.0 minutes made little difference in the total amount of

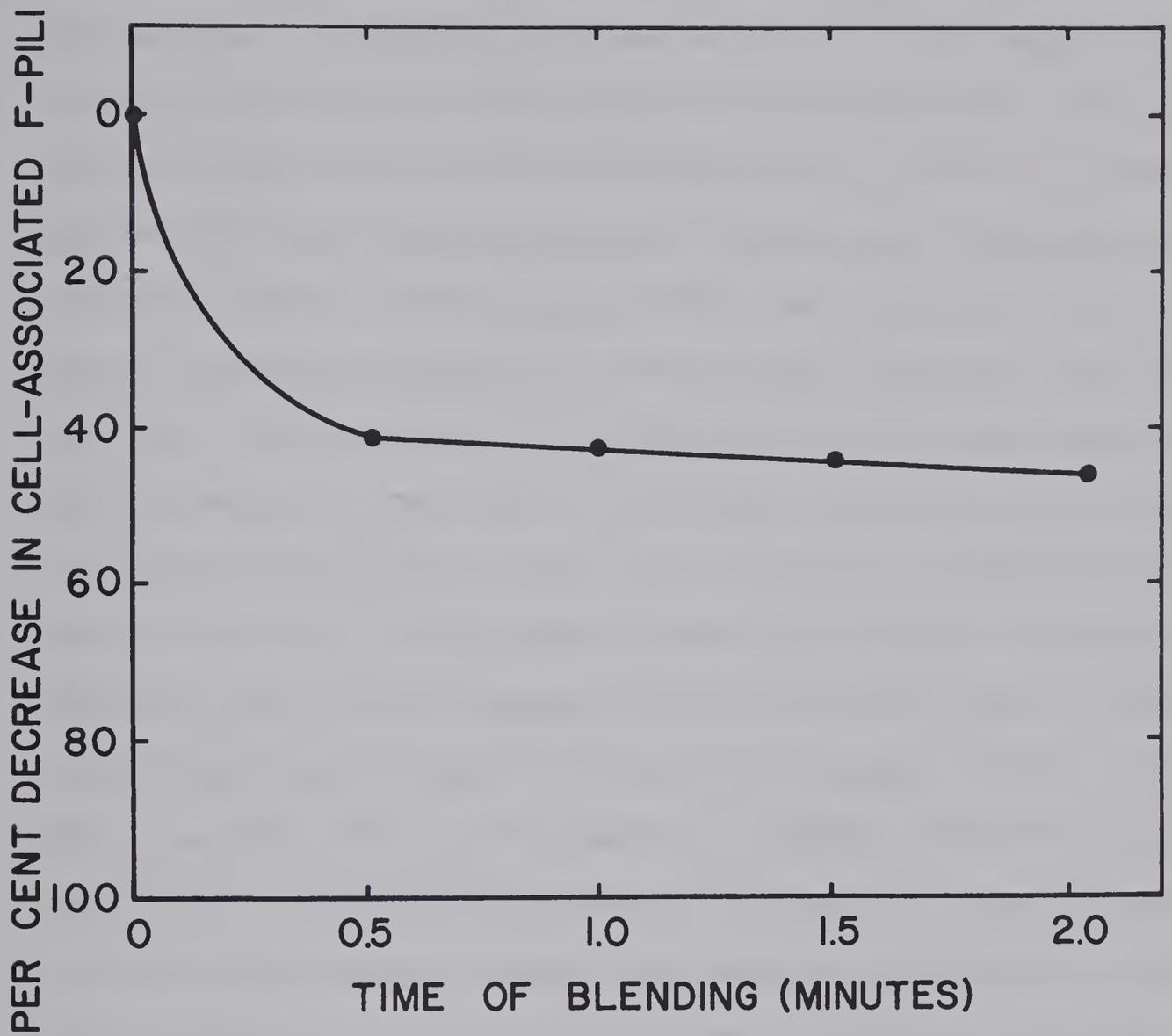


Figure 4.3. Decrease in cell-associated F-pili as a function of time of blending.

HB11 cells were blended at 2500 revolutions per minute for the various time periods indicated. Cultures were blended at 4° in Z broth medium containing 0.40 M formaldehyde. Values plotted represent the average of at least 5 measurements. All values represent cell-associated F-pili only.

cell-free F-pili recovered, a duration of 0.5 minute was chosen as a minimal and sufficient time of blending to remove F-pili from cultured HB11 bacteria. Unfortunately, no studies related to the length of F-pili recovered from cultures of HB11 bacteria (blended for various time intervals at 2500 r.p.m.) could be undertaken due to a lack of an operational departmental electron microscope at the time the above experiments were performed. However, Novotny et al. (1969a) have shown that F-pili are sheared into smaller fragments by about the same speeds that remove them from cells. This observation is of importance in filtration assays in which the amount of free F-pili is determined indirectly by the binding of radioactive male phage to pili, and by retention of F-pili-phage complexes by a filter. If high speed blending were employed for extended periods of time, the pili fragments would be relatively small and some of these might pass through the filter (Novotny et al., 1969a). In fact, Brinton and Beer (1967) and Danziger and Paranchych (1970) have shown that the male phage-adsorbing capacity of supernatant fluids, as measured by the filtration assay, decreases when cultures are subjected to high speed blending for extended periods of time (≧2 minutes of blending). This suggests that the F-pili were sheared into small pieces that passed through filters or that the F-pili were affected in some other way so that the phage could no longer attach to them (Novotny et al., 1969a).

(3) Determination of the Cell Density of HB11 Bacteria at Which F-Piliation is Maximal

To maximize the yields of long, intact F-pili obtained from cultures of HB11 bacteria (blended under the conditions previously described), it was necessary to ascertain the point of maximal F-piliation in this strain of bacteria. It should be noted that "F-piliation" here refers to a

distribution of lengths of outgrown cell-associated F-pili present in a bacterial culture. The point of maximal F-piliation in the HBl1 strain of E. coli was determined as follows. Cultures of HBl1 bacteria were grown in Z broth medium to cell densities of 2.5×10^8, 5.0×10^8, 1×10^9, and 2×10^9 (approximate) bacteria/ml. Bacteria were removed from each culture by differential centrifugation at 8000 x g for 10 minutes, and the cell pellets were then resuspended to their original concentration in fresh Z broth containing 0.40 M formaldehyde. ^{32}P-labeled R17 phage was added to each culture at an input of 1000 particles per bacterium, and the phage allowed to attach to the pili for 40 minutes at 4°. After this time, the c.p.m. attached per bacterium were tabulated, and the resultant values plotted against the correct cell density of bacteria. This curve is shown in Figure 4.4. It should be noted that a linear relationship of phage attachment vs. cell concentration (as shown in Figure 3.3), at an input ratio of 1000 p/c, is obtained with HBl1 cultures as high as 2×10^9 bacteria/ml in cell density.

The curve in Figure 4.4 indicates that maximal F-piliation of HBl1 bacteria occurs during the early log-phase stage of growth (2×10^8 to 4×10^8 bacteria/ml cell density). This observation was not entirely surprising as maximal mating pair formation (Novotny et al., 1969b; Ou and Anderson, 1970), and penetration of R17 phage nucleic acid (Krahn, 1971), occur at early log-phase. As bacterial cell density increased, the amount of cell-associated F-pili (measured indirectly by counts per minute attached per bacterium) decreased continually. This trend was most perceptible between the cell densities of 2.5×10^8 and 5.0×10^8 bacteria/ml, where it was found that the amount of F-pili in a culture of HBl1 bacteria at 5.0×10^8 cells/ml was only 43% of the amount of

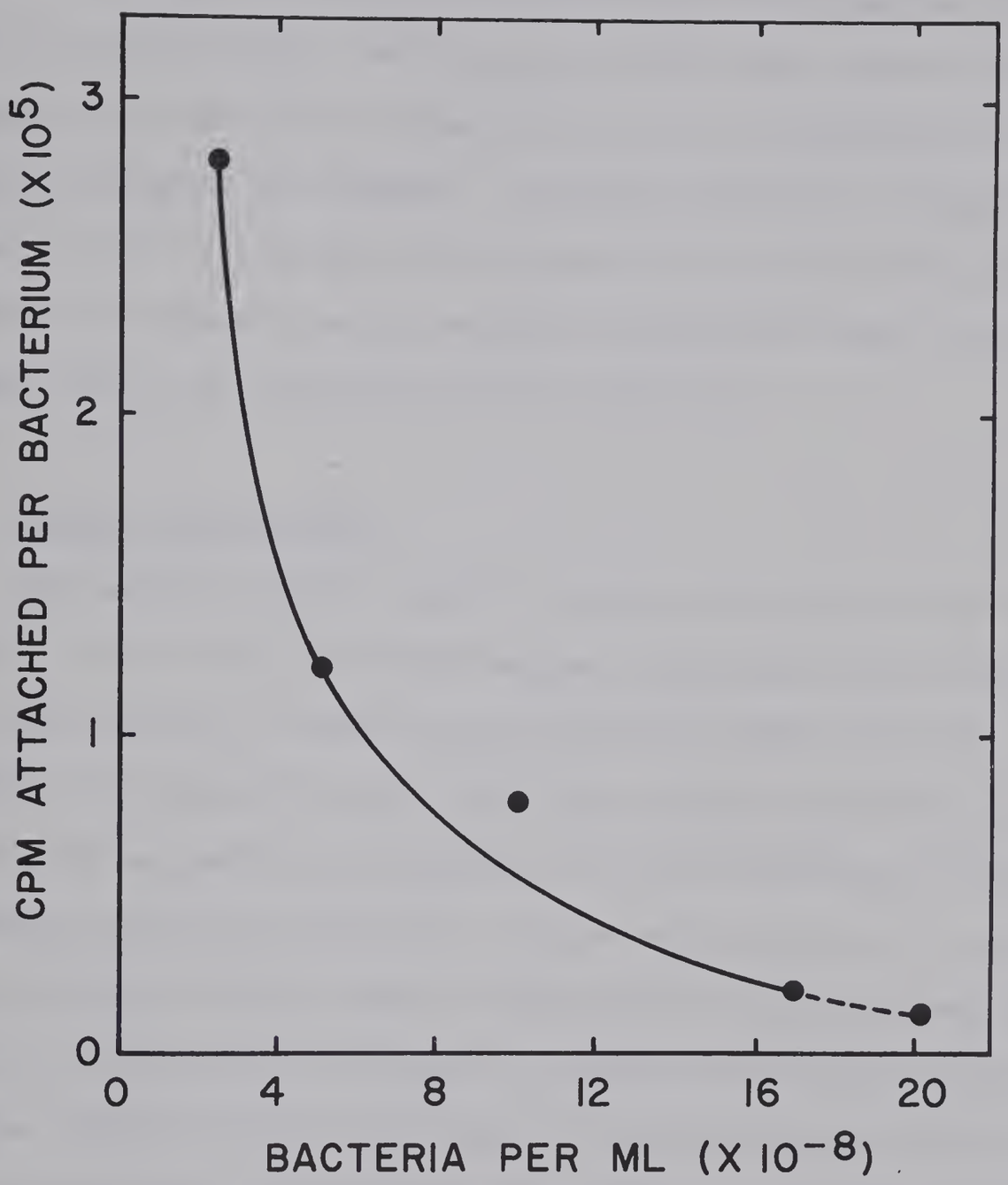


Figure 4.4. Extent of F-piliation in cultures of HB11 bacteria at varying cell densities.

Cultures of HB11 bacteria were grown in Z broth medium at 37° to the cell densities indicated. Bacterial cells were then removed from suspension and assayed for attached F-pili. F-piliation was measured indirectly as counts per minute attached per bacterium. All values plotted represent the average of at least 5 measurements. Only cell-associated F-pili are represented.

F-pili in an identical culture at 2.5 x 10^8 cells/ml. The quantity of cell-associated F-pili at a bacterial density of 2 x 10^9 cells/ml was 4% of the amount of bacterial-attached F-pili in an identical culture at 2.5 x 10^8 cells/ml. Novotny et al. (1969b) have suggested that F-pili outgrowth, mating and male phage infection are all regulated by the same aerobic metabolic requirements. As aerobic metabolism presumably slows down in cells approaching late log-phase and stationary phase growth, because of a competition for existing nutrients and oxygen, one would expect this to be reflected in F-pili production.

C. SUMMARY AND DISCUSSION

Two methods, commonly used for studying the removal of bacterial pili from host cells, have been compared—separately and in combination—for their ability to remove F-pili from cultured HB11 bacteria. Natural release, the process by which F-pili are separated from host cells by continuous outgrowth and "dropping off" of the structures, and mechanical shearing (blending) of bacterial cultures were both shown to remove about equal amounts of F-pili under the experimental conditions used, whereas removal of F-pili by both methods in combination was essentially cumulative. Natural release alone gave 30 to 50% conversion of all F-pili into the cell-free form, depending upon the growth medium used. Blending of bacterial cultures per se (2500 r.p.m. for 30 seconds) converted about 45% of the total F-pili population into the cell-free state regardless of the culture medium employed. A combination of both methods resulted in the combined removal of 65-70% of all F-pili into a cell-free form. That essentially all the F-pili released in this manner were detectable by the standard phage assay procedure (Table 5.2) indicates that they were

sufficiently undegraded to be retained on cellulose triacetate filters.

Previous experimentation (Novotny et al., 1969a) has shown that a range of blending speeds, extending from the beginning of removal to complete removal, is characteristic for each bacterial surface appendage. The curve for removal of an appendage vs. blender speed is called the "blending spectrum for removal", and may be concisely described either by the speed that removes 50% of the appendages, or by the width of a speed range between which two arbitrarily-chosen fractions of F-pili are removed (e.g., the 20 to 80% range suggested by Novotny et al., 1969a). The blending spectrum for removal of HB11 F-pili had a midpoint of 3200 r.p.m., and a 20 to 80% removal range of 2500 to 7000 r.p.m. Novotny et al. (1969a) have shown that the blending spectra for removal of F-pili and reduction in length of the appendages are nearly identical. This finding suggests that F-pili are sheared into fragments by about the same speeds which remove them from cells. Present evidence shows that substantial fragmentation of F-pili occurs at blending speeds in excess of 3000 r.p.m. (see Plate 4.1).

Studies of F-pili outgrowth (Brinton, 1965; Brinton and Beer, 1967; Brinton et al., 1964; Novotny et al., 1969a) have shown that these structures reappear very rapidly after blending, reaching a characteristic length of 1.2 to 1.3 μm after 4 minutes from onset of removal, and remaining at this length thereafter. This pattern of outgrowth is quite different from that of flagella or Type I (common) pili which increase in length throughout the generation time of the cell. The most probable explanation for this pattern of F-pili outgrowth is that F-pili are rapidly appearing on the cell surface and just as rapidly disappearing from it. Support for this view is found in the relatively narrow distribution of

lengths of these appendages, the large numbers of free F-pili found in the culture medium during cell growth, and the fact that large amounts of F-pili are mechanically removed only by an amount of agitation much greater than that used to grow cultures. This mode of outgrowth—i.e., outgrowth to a certain length and then separation from the cell—is similar to that of the DNA male phages and is consistent with a virus-like structure for F-pili, as originally proposed by Brinton in 1965 (Novotny et al., 1969a).

In conclusion, removal of F-pili from HBl1 bacteria by a combination of blending and natural release appears to provide the most efficient means of removing F-pili from cells for purification purposes, resulting in the overall removal of approximately 60 - 70% of these structures.

CHAPTER V

THE PREPARATION OF PURIFIED F-PILI

This chapter describes the general procedure by which F-pili were removed from large volumes of bacterial culture, separated from contaminant cellular material, and purified to an extent suitable for chemical analysis. As E. coli HB11 is a bacterium possessing an average of one F-pilus per cell (Novotny et al., 1969a), large volumes of bacterial culture were required to obtain sufficient amounts of F-pili material for further studies. The preparative method to be described was so designed that one person, in a maximum of 5 average working days, could completely process and purify F-pili material from 30 litres of culture supernatant.

A. PRESERVATION OF F-PILI BY THE THIOL-REDUCING AGENT, 2-MERCAPTOETHANOL

To maintain constant levels of assayable HB11 F-pili during their isolation from host cells, and subsequent purification, a thiol-reducing agent, 2-mercaptoethanol, was introduced into F-pili suspensions. The intent of this operation was to prevent spontaneous oxidation of disulfide linkages which might exist in the F-pili protein. As 2-ME is commonly used to maintain monothiols and disulfides in the reduced state (Gordon, 1969), and since protein is a known component of F-pili (Brinton, 1965), it was anticipated that F-pili preparations might better retain their biological activity in the presence of this agent. To test this hypothesis, the following experiment was performed.

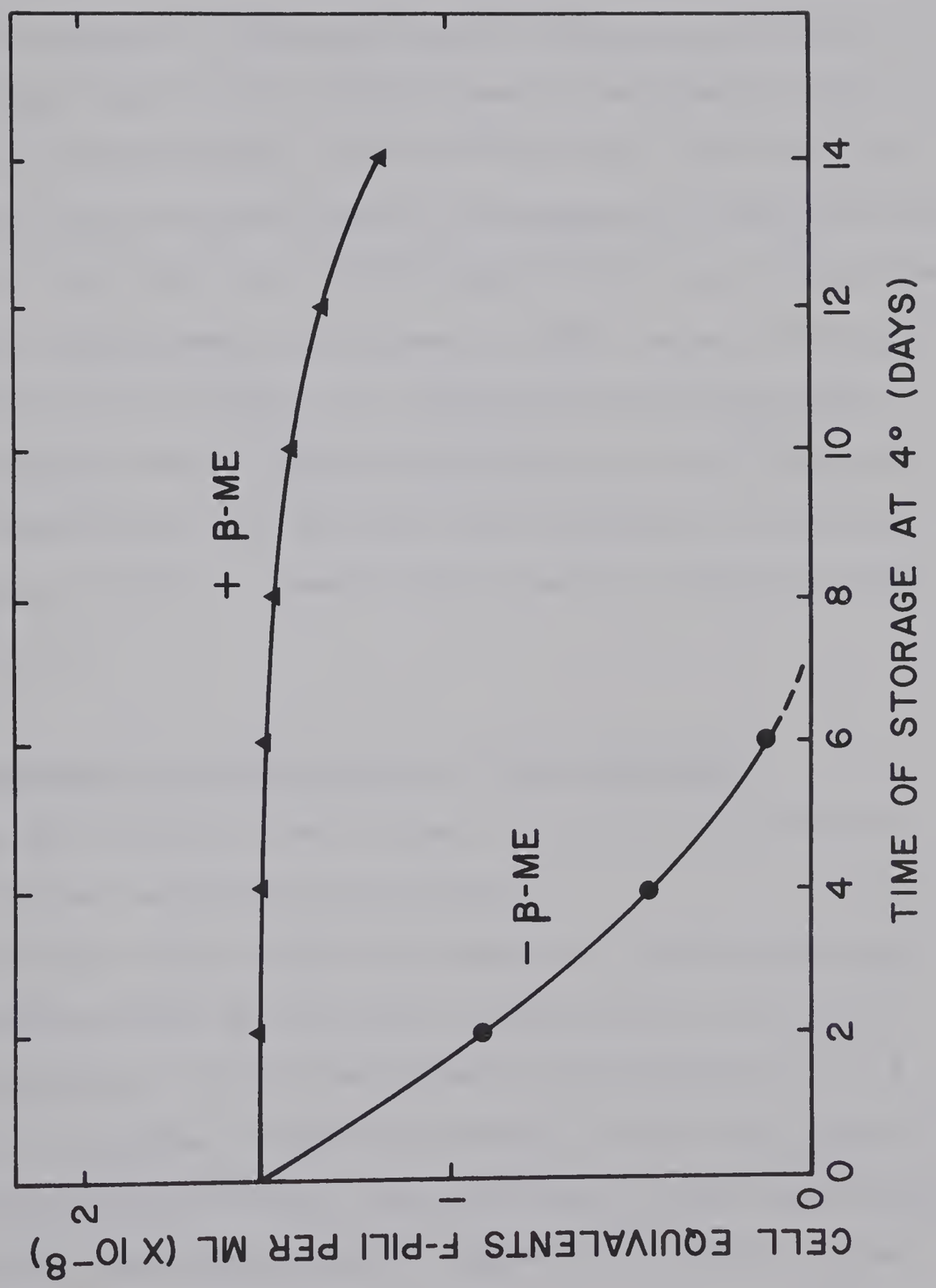
An F-pili survival study was conducted over a period of two weeks on two identical suspensions of newly isolated, "purified", cell-free

F-pili. The F-pili material was obtained from preparations made according to the procedure described in Section B of this chapter. Suspensions of F-pili (1.5 x 10^8 CE/ml in basic TMM) were maintained under airtight conditions at 4° in 50 ml culture flasks sealed with parafilm. One flask contained 2-ME (final concentration = 6 mM), a second flask contained no 2-ME. At two day intervals, 1 ml aliquots were withdrawn from each vessel and assayed for F-pili by the procedure described in Chapter III. The results of this experiment are shown in Figure 5.1. It was found that the phage-attachment activity of F-pili decreased rapidly in the absence of 2-ME, and was completely absent 7 days post-incubation. In contrast, F-pili material incubated in the presence of 2-ME (final concentration = 6 mM) showed a much slower decline in phage-adsorption capability with 80% of the original biological activity remaining after the 2 week incubation period had elapsed. As these results showed that 2-ME was capable of temporarily preserving F-pili biological activity *in vitro*, a parallel experiment was conducted to determine if greater amounts of F-pili could be isolated from cultured bacteria grown in the presence of this agent. If such evidence could be obtained, it would provide a means of increasing the yields of F-pili.

Two 30-litre cultures of *E. coli* HB11 bacteria were grown in TSB at 37° to 4 x 10^8 cells/ml, as described in Section B of this chapter. One 30-litre volume contained 2-ME (final concentration = 6 mM) during the growth phase, while the second volume was grown in the absence of this agent. When the cultures reached the desired cell density, cell growth was arrested by the addition of formaldehyde to 0.40 M, and both cultures were chilled to 4°. F-pili were removed from host cells by blending, and purified from contaminant cellular material (see adjacent

Figure 5.1. Loss of biological activity by cell-free F-pili incubated at 4° in basic TMM in the presence and absence of 2-ME.

Suspensions of F-pili (1.5×10^8 CE/ml in basic TMM) were incubated in air-tight vessels at 4° in the presence and absence of 2-ME (final concentration = 6 mM). The phage attachment capability was assayed at 2 day intervals, as described in the text.


CELL EQUIVALENTS F-PILI PER ML (X 10^{-8})
2
1
0
+ β-ME
- β-ME
0
2
4
6
8
10
12
14
TIME OF STORAGE AT 4° (DAYS)

Section B), after which purified preparations were immediately assayed for F-pili content. Table 5.1 summarizes the recovery data obtained by this procedure.

Yields of "pure" F-pili recovered from cultures of HB11 bacteria grown in the presence of 2-ME were invariably higher than yields of F-pili obtained from identical cultures grown in the absence of this agent. In the present example, 4.08×10^{10} CE "pure" F-pili were obtained from a 30 litre culture grown in the presence of 2-ME (final concentration = 6 mM) while 1.62×10^{10} CE "pure" F-pili were obtained from an identical culture grown in the absence of 2-ME. As each culture was treated identically throughout the isolation and purification steps, one must conclude that (a) either 2-ME serves to activate F-pili synthesis in some way, or, providing that F-pili synthesis is unaffected by this agent, that (b) it prevents F-pili breakdown during the purification procedure.

B. THE PREPARATION AND PURIFICATION OF F-PILI CONCENTRATES

The general procedure used to prepare and purify F-pili from 30 litres of culture supernatant was as follows.

Two 15-litre volumes of TSB were prepared in 5 gallon carboys and autoclaved for one hour at 121° under a steam pressure of 20 lbs/in^2. After sterilization, the media was allowed to equilibrate to 37° in a warm room. An inoculum (15 ml) of an overnight culture of *E. coli* HB11 was introduced into each carbouy, after which $MgCl_2$ (final concentration = 5 mM) and 2-ME (final concentration = 6 mM) were also added. The cultures were aerated at 37° until the cell density reached 4×10^8 bacteria/ml; foam control was achieved by occasionally spraying the surface of the

Table 5.1

Yields of Purified F-Pili Recovered from 30 Litre HB11 Cultures Grown in the Presence and Absence of 2-ME (See Text for Details)

Culture Conditions	Total µg F-Pilin Recovered	Specific Activity of F-Pili (CE/µg F-pilin)	Total CE F-Pili Recovered
Growth + 2-ME	68	6×10^8	4.08×10^{10}
Growth - 2-ME	162	1×10^8	1.62×10^{10}

cultures with silicone. Cell growth was then arrested by adding formaldehyde to each 15 litre volume to 0.40 M, and chilling the carboys to 4°.

F-pili were sheared from host cells at 2500 r.p.m. by passing chilled, formaldehyde-fixed cultures through a continuous-flow Omni-Mixer blending apparatus. Cultures were fed through a specially-designed, ice-cooled, blending cup of 200 ml capacity (Figure 5.2) at a flow rate of 80 ml/minute. The change from manual to continuous-flow blending was made to expedite the removal of F-pili from the very large volumes of bacterial culture used. Identical amounts of F-pili were removed using either the manual or continuous flow technique (see Chapter IV, B, and Table 5.2). After blending, cells were harvested by differential centrifugation at 8000 x g for 10 minutes, and the supernatant phase decanted off into 4 litre beakers. Cell-free supernatant F-pili were then removed from suspension by acidifying the culture medium to pH 4 with HCl, and allowing F-pili to precipitate at 4° for 12 hours, as originally described by Brinton and Beer (1967).

The F-pili precipitate was collected from the pH 4 supernatant by differential centrifugation at 8000 x g for 10 minutes in 250 ml plastic centrifuge bottles. After harvesting the entire F-pili precipitate, it was resuspended in 50 ml of basic TMM containing 2-ME (final concentration = 6 mM), pH 10.5, and dialyzed against identical buffer for 20 hours at 25°. In this way, F-pili were separated from non-F-pili cellular material. The dialyzed suspension was clarified by differential centrifugation at 10,000 x g for 10 minutes, and the F-pili enriched supernatant removed. This supernatant was then adjusted to pH 4 with 10 N HCl to reprecipitate F-pili. After a 10 minute exposure to acid conditions,

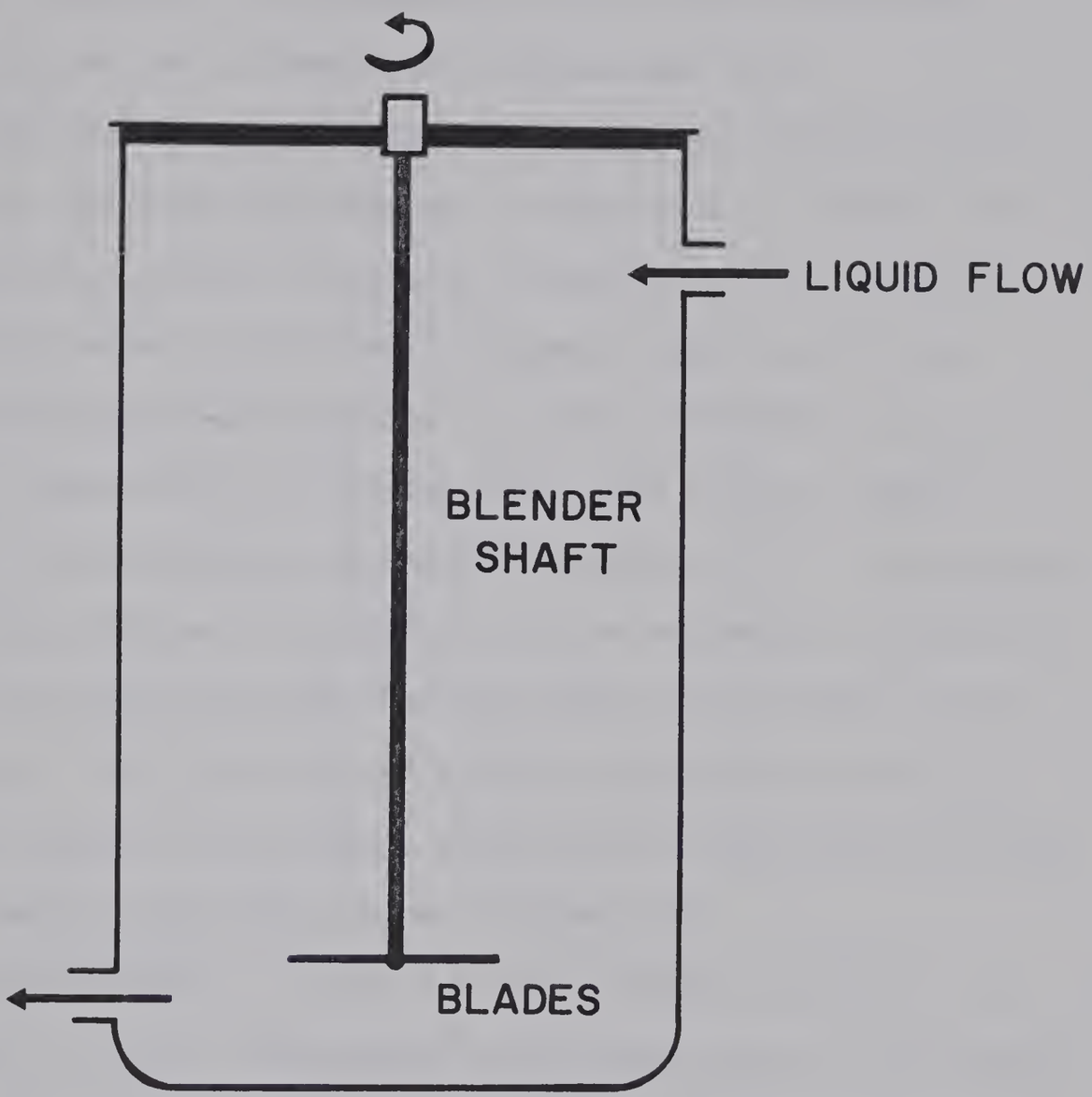


Figure 5.2. Diagrammatic representation of the continuous-flow blender cup used to shear F-pili from HB11 bacteria.

A normal Omni-Mixer blender cup was modified for continuous-flow operation by welding inlet and outlet portals into the steel vessel as shown. Flow rate of the culture was 80 ml/minute. Blending speed was 2500 r.p.m. The entire system was initially filled with culture, after which the flow was initiated and maintained by gravity. See text for further details.

the F-pili were concentrated by differential centrifugation at 10,000 x g for 10 minutes, and the pellet material (herein termed "crude" F-pili) resuspended in 10 ml of basic TMM containing 2-ME (final concentration = 6 mM), pH 7.4, in preparation for further purification. The suspension was now a translucent, light-greyish color.

The final step in the purification of F-pili was banding by isopycnic density gradient centrifugation. Suspensions of "crude" F-pili were adjusted to a specific gravity of 1.24 gm/cm^3 by the addition of an appropriate amount of solid CsCl. Isopycnic gradients were then formed by spinning the salt solution at an RCF of 105,000 x g for 40 hours in a Spinco SW 50.1 titanium rotor using a Spinco Model L preparative ultracentrifuge (temperature of chamber = 4°). Centrifuged gradients were collected in accordance with the methodology outlined in Chapter II, and, after dilution with basic TMM containing $MgCl_2$ (final concentration = 5 mM), were assayed for phage adsorption activity to identify the region of the gradient containing the experimental material. A typical banding profile is provided in Figure 5.3.

The buoyant density of purified F-pili invariably was found to be in the range of 1.240 to 1.245 gm/cm^3 with a second peak at 1.197 gm/cm^3. The heavier F-pili (ρ = 1.240 - 1.245 gm/cm^3) were found to be an association product of F-pili and F-pili-specific carbohydrate whereas the lighter F-pili (ρ = 1.197 gm/cm^3) were composed entirely of protein (see Chapter VI for further details). The latter buoyant density value is in agreement with a previous report by Ippen (1967), who examined HB11 F-pili under much the same conditions presented above. She notes that HB11 F-pili have a mean buoyant density (at 25°) in CsCl of 1.197 gm/cm^3, but that with other bacterial strains, phage adsorption peaks at higher

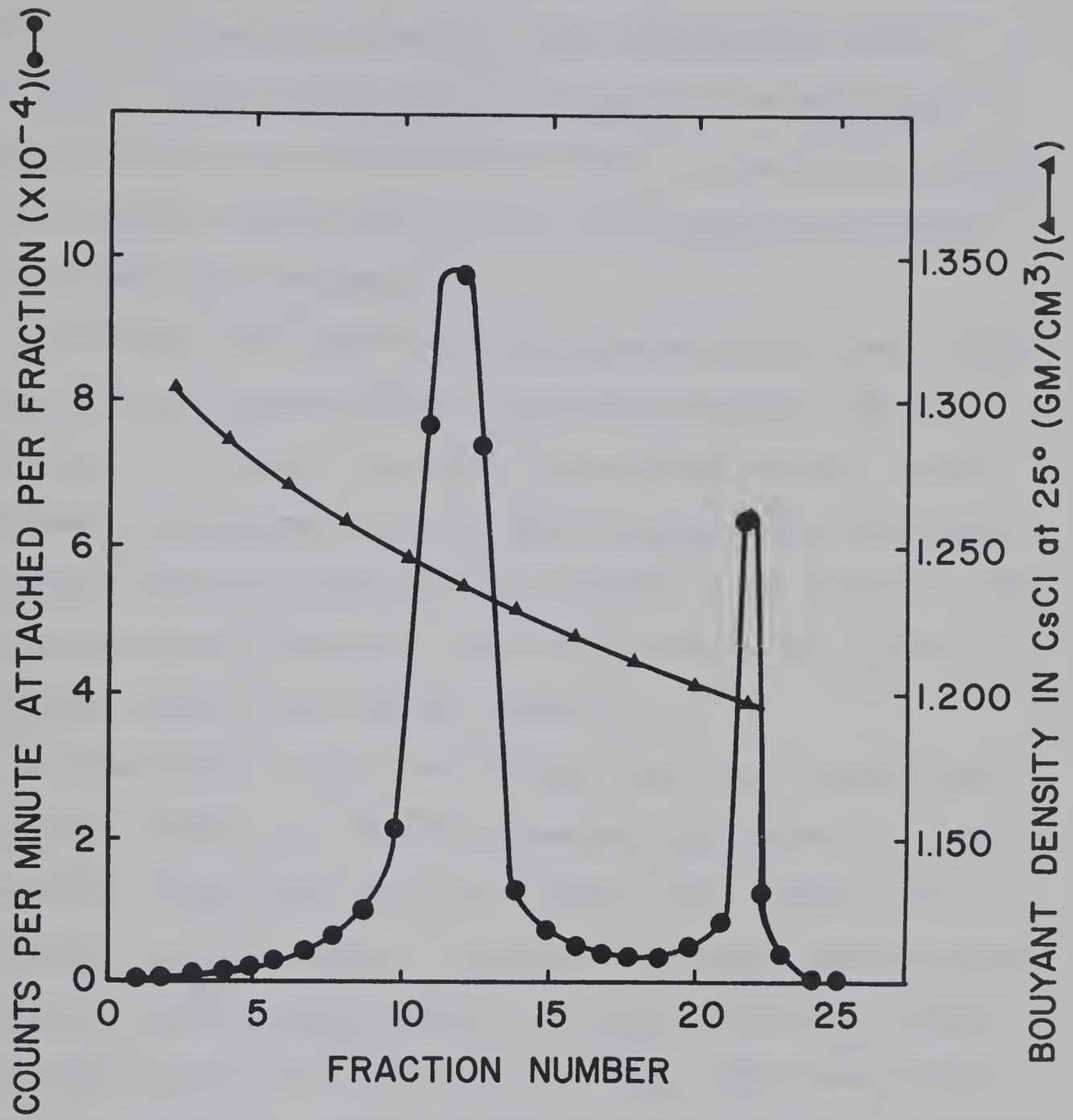


Figure 5.3. Banding profile of isopycnically-separated "pure" F-pili in a CsCl gradient.

CsCl isopycnic gradients of "purified" F-pili material were collected in 12 drop fractions, and the buoyant density of each fraction determined by refractive index measurements at 25°. Each fraction was then diluted by the addition of 1 ml basic TMM, pH 7.4, containing 2-ME (final concentration = 6 mM), $MgCl_2$ (final concentration = 5 mM) and 2.5 x 10^{12} particles ^{32}P-labeled R17 phage (1 x 10^6 c.p.m.). Phage attachment was allowed to proceed for 40 minutes at 4°. The fractions were assayed for attached c.p.m. as described in Chapter III.

densities have been observed. She further explains that these peaks probably result from F-pili complexes with other material such as Type I pili. Since no Type I pili were present in the HB11 strain of E. coli used for the experiments in this thesis, it was quite surprising to find a heavier banding peak of F-pili with associated carbohydrate in the present F-pili preparations.

Both "heavy" and "light" F-pili fractions were collectively pooled and exhaustively dialyzed against 4° basic TMM containing 2-ME (final concentration = 6 mM), pH 7.4, for 12 hours. After dialysis, F-pili were removed from suspension by acid (pH 4) precipitation, and concentrated by differential centrifugation at 10,000 x g for 10 minutes. The pellet material, now containing "pure" F-pili, was further analyzed for contaminant material (see adjacent Section C).

A balance sheet of F-pili recoveries at each step of purification is provided in Table 5.2. The data presented therein show the average recoveries of F-pili from a 30 litre culture of HB11 bacteria grown to a density of 4×10^8 cells/ml. It will be noted that between steps (g) through (i) in the outlined procedure, a very sharp decrease in F-pili yield (53% of control to 6.3% of control) occurs. The reason for this decrease is unknown at present, although it is possible that acid (pH 4) precipitation of F-pili from step (g) to form step (i) material may be detrimental to concentrated F-pili. A further decrease in F-pili yield occurs as "crude" F-pili in step (i) are concentrated by CsCl density banding to form the "pure" F-pili of step (j). This loss of F-pili can, in large part, be attributed to prolonged exposure to the high salt environment of CsCl gradients, which seems to split the protein and carbohydrate moieties of "heavy" F-pili from one another with concomitant

Table 5.2

Tabulation of F-Pili Recoveries at Each Step of Purification
(See Text for Details)

Step	Culture Component	Total Volume (ml)	Total Cell-Equivalents F-Pili Recovered	% Recovery of Control
a	Intact culture	3×10^4	1.2×10^{12} (control)	100
b	Blended culture	3×10^4	1.16×10^{12}	97
c	Supernatant (cell-free F-pili)	3×10^4	7.8×10^{11}	65
d	Bacterial pellet (resuspended)	1×10^3	3.8×10^{11}	32
e	pH 4 F-pili-free supernatant	3×10^4	3.0×10^{10}	2
f	F-pili pellet (resuspended)	50	7.5×10^{11}	63
g	Dialyzed F-pili suspension	100	6.3×10^{11}	53
h	Resuspended cell. ppt.	100	1.2×10^{11}	10
i	"Crude" F-pili	10	7.5×10^{10}	6.3
j	"Pure" F-pili	2	2.0×10^{10}	1.7

loss of biological activity (see Chapter VI for details). As acid (pH 4) precipitation and CsCl banding are necessary techniques in the present purification procedure, the observed losses of F-pili at the above mentioned steps were found to be unavoidable.

C. CHEMICAL PURITY OF F-PILI CONCENTRATES

To determine whether or not isolated F-pili were sufficiently pure to undergo chemical analysis, two experiments were performed whereby the extent of F-pili separation from contaminant non-F-pili cellular material was determined. These experiments, in conjunction with electron microscopic, biological activity, and CsCl isopycnic gradient centrifugation observations, served as the basis for determining F-pili homogeneity.

It was reasoned that one of the simplest and most direct means of obtaining information on the purity of recovered F-pili would be to examine the stepwise removal of a universally-incorporated, highly radioactive label from a small volume culture of HB11 F^- bacteria mixed with a large volume culture of HB11 F^+ bacteria containing no radioactive label. Since the F^- strain employed would differ from the wild-type parent only in the lack of F-pili, the results obtained would indicate the sequential removal of all compounds not directly associated with assembled, cell-free F-pili. The percent recovery of isotopic label, at any step of purification, would reflect, then, the degree of F-pili purity at that step.

A 1050 ml culture of HB11 F^+ bacteria was grown normally in trypticase soy broth at 37° to density of 4×10^8 cells/ml, as previously described. When the culture reached the desired cell density, cell growth

was arrested by the addition of formaldehyde to 0.40 M, and chilling of the culture to 4°. A second, 100 ml culture of HB11 F^- bacteria was grown at 37° in synthetic glucose TMM medium containing universally-labeled glucose (0.5 mCi ^{14}C-(U)-glucose; 3.0 mCi/mmole) to a density of 4 x 10^8 cells/ml under identical conditions imposed upon the first culture. When the labeled culture reached the desired cell density, cell growth was again halted by formaldehyde treatment, and the culture chilled to 4°. Both cultures were then pooled, and the collective mixture treated for the isolation and purification of F-pili, as described in Section B, above. Results of ^{14}C-labeled glucose-incorporated radioactivity recovered at each step of purification are summarized in Table 5.3. It was found that final CsCl isopycnically-banded F-pili were greater than 99% free of the radioactivity present in the starting mixed culture of ^{14}C-labeled F^- cells and non-radioactive F^+ cells. The residual label in step (j), some 25,000 c.p.m., was found to be an unfortunate carryover in this procedure; since it represented much less than 1% of the total input level of radioactivity in step (a), or about 1% of the c.p.m. associated with the F-pili "pellet" material in step (f), it was considered non-specific and was not traced further. Step (f) was chosen as a reference point for comparison of c.p.m. recoveries in the subsequent steps (g) - (j) because at this point one had, in concentrate form, the net amount of cell-free F-pili on which eventual purification was performed. It is possible that the 25,000 c.p.m. found at step (j) were contributed by traces of unutilized ^{14}C-labeled glucose.

To further investigate the extent to which "pure" F-pili were free of contaminant cellular material, F-pilin, the protein component

Table 5.3

Removal from F-Pili of ^{14}C-Glucose-Labelled F^- Cell-Material During the Purification Procedure (See Text for Details)

Step	Culture Component	Total Volume (ml)	Total ^{14}C c.p.m.	% Recovery of Control c.p.m.
a	Intact culture	1150	4.55×10^8 (control)	100
b	Blended culture	1150	4.60×10^8	100
c	Supernatant (cell-free F-pili)	1150	1.48×10^8	32.2
d	Bacterial pellet (resuspended)	1150	3.12×10^8	67.8
e	pH 4 F-pili-free supernatant	1150	1.46×10^8	31.8
f	F-pili pellet (resuspended)	31.5	2.00×10^6	0.43
g	Dialyzed F-pili suspension	29	8.15×10^5	0.18
h	Resuspended cell. ppt.	5	1.18×10^6	0.25
i	"Crude" F-pili	5.3	3.47×10^5	0.075
j	"Pure" F-pili	2	2.50×10^4	0.0054

of F-pili, was isolated from "crude" and "pure" F-pili concentrates in subunit form, and electrophoresed in 10% SDS-polyacrylamide gels (see Chapter II for details). Resolution of F-pilin in this system is shown in Figures 5.4 and 5.5. Examination of the figures shows that both "crude" and "pure" F-pilin were composed of two closely migrating protein subunit species. This finding is supported by numerous visual observations of stained SDS-polyacrylamide gels containing F-pili material, in which two very sharp coelectrophoresing bands were noted which could not be resolved distinctly by gel tracing due to their closeness to one another. Aside from the two peaks described, "crude" F-pili preparations contained slight traces of other contaminant proteins (see Figure 5.4) which were much reduced, or totally absent, in gel profiles of "pure" preparations. From the foregoing data, it was assumed that "pure" F-pili were sufficiently pure to be analyzed by chemical means.

D. SUMMARY AND DISCUSSION

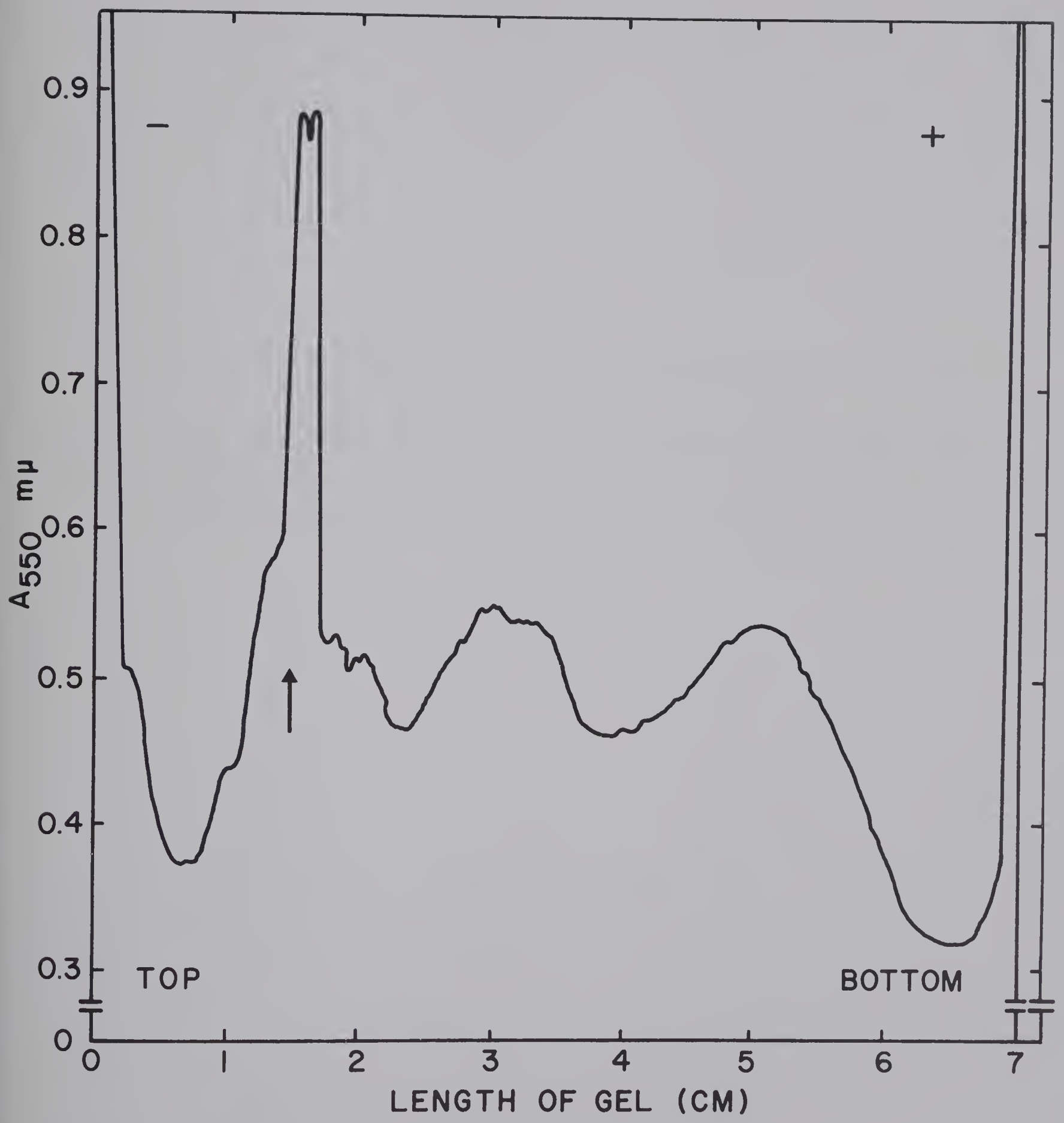
Several interesting features related to the purification of F-pili have emerged from studies described in this chapter.

(1) A comparison of cell-free F-pili yields, obtained from cultures of HB11 bacteria grown in the presence and absence of 2-ME, has shown that this agent increases total levels of recoverable F-pili (see Table 5.1). F-pili phage adsorption activity was kept constant, *in vitro*, for 8 to 10 days by this agent, whereas the phage-absorption activity of control, untreated F-pili declined to zero after 7 days from isolation (Figure 5.1).

(2) F-pili concentrates have been made by acid precipitating cell-

Figure 5.4. SDS-polyacrylamide gel electrophoresis of F-pilin obtained from concentrates of "crude" F-pili.

Total protein from concentrates of "crude" F-pili (200 µg/ml) was solubilized, electrophoresed, and analyzed for by protein staining, as described in Chapter II. Electrophoresis was at 5 mA/column for 2.5 hours. The marker arrow indicates the position of F-pilin. See text for further details.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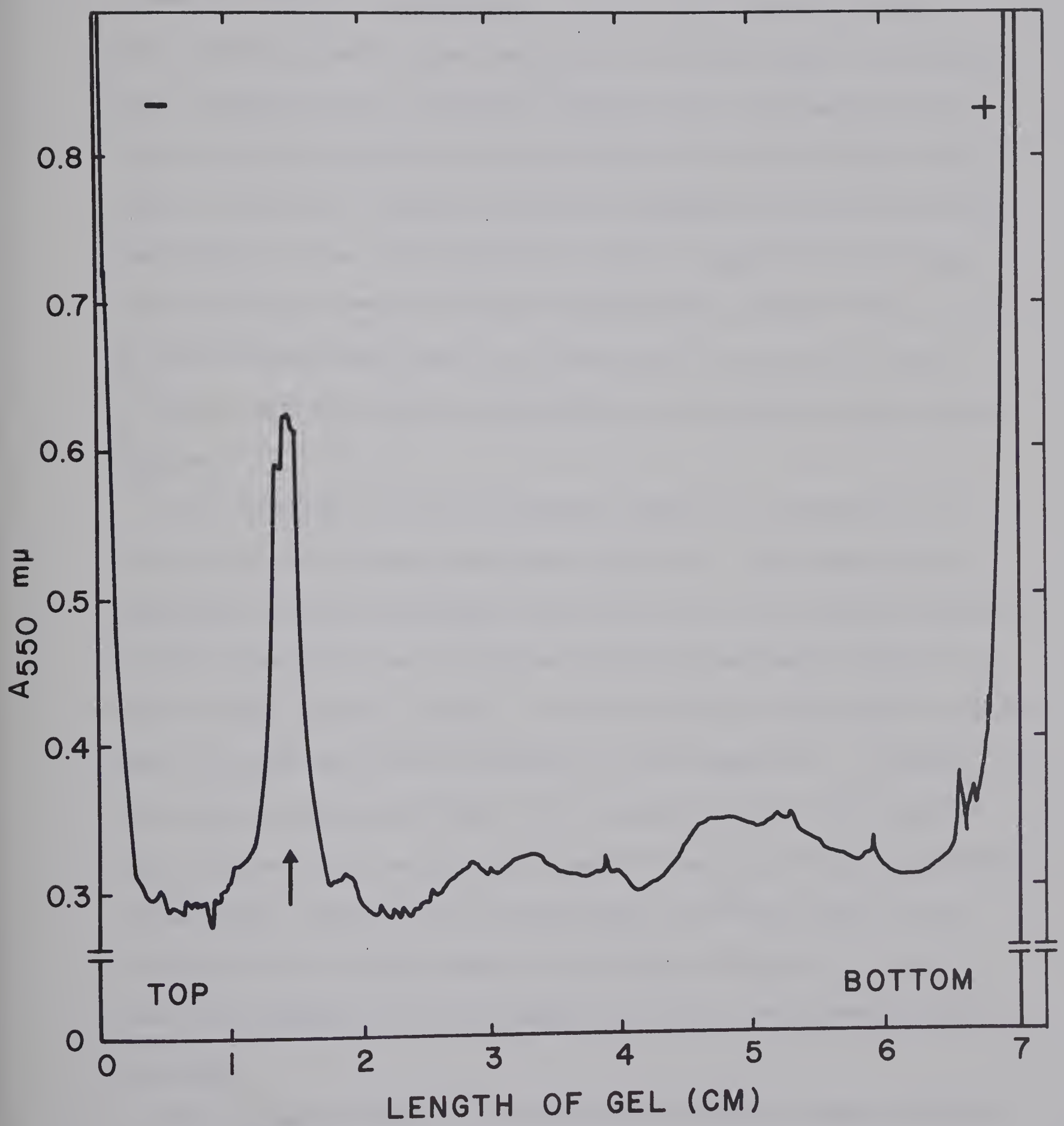
7
LENGTH OF GEL (CM)

Figure 5.5. SDS-polyacrylamide gel electrophoresis of F-pilin obtained from concentrates of "pure" F-pili.

Total protein from concentrates of "pure" F-pili (170 µg/ml) was solubilized, electrophoresed, and analyzed for by protein staining, as described in Chapter II. Electrophoresis was at 5 mA/column for 2.5 hours. The marker arrow indicates the position of F-pilin. See text for further details.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LENGTH OF GEL (CM)

free F-pili from 30 litre volumes of bacterial culture supernatant, partially eliminating non-F-pili cellular material by extensive dialysis against basic TMM, pH 10.5, and further purifying the F-pili by CsCl isopycnic gradient centrifugation. F-pili prepared in this manner were observed to have buoyant densities (at 25°) of 1.240 - 1.245 gm/cm^3 with a second peak at 1.197 gm/cm^3 (Figure 5.3). The "heavier" F-pili were an association product of both F-pilin and F-pili-specific carbohydrate whereas the "lighter" F-pili were composed entirely of protein (see Chapter VI for further details). From a compilation of the stepwise recoveries of F-pili during the purification process (Table 5.3) it was found that only about 2% of the control, total culture level of F-pili reach the stage of separation by CsCl density gradient centrifugation.

(3) "Purified" F-pili concentrates appear to be homogeneous on the basis of the following preliminary analyses. Three qualitative procedures—electron microscopy (Plates 3.1, 3.2, 4.1), isopycnic banding in CsCl (Figure 5.3), and biological activity measurements (Table 5.2)—have provided indirect evidence for F-pili purity in that samples examined seem to contain very little extraneous cellular material. A rigorous isotope dilution experiment (Table 5.3) revealed that final CsCl isopycnically-banded F-pili were greater than 99% free of non-F-pili-associated radioactivity. Examination of F-pilin from "crude" and "pure" F-pili concentrates by acrylamide zone electrophoresis (Figures 5.4, 5.5) revealed the presence of two slow-migrating closely electrophoresing protein peaks.

The foregoing observations have shaped, roughly, a basic procedure whereby F-pili can be gathered from large volumes of bacterial culture

and subsequently purified for chemical analysis. The finding that 2-ME stabilizes F-pili *in vitro*, and possibly *in vivo*, is an effective means of increasing yields of experimental material. It will be noted that a sharp drop in F-pili recovery occurs at steps (h) through (j) in the purification procedure (Table 5.2). The reason for this decline is only partially understood at present; the high salt environment of CsCl gradients apparently degrades some of the "heavier" F-pili by splitting the protein and carbohydrate moieties from one another with concomitant loss of F-pili biological activity (see Chapter VI for details). The dual nature of F-pili buoyant density, a subject dealt with in the next chapter, can, at present, be simply explained as the presence of two types of F-pili—one with associated carbohydrate and one without associated carbohydrate—in cell-free F-pili concentrates. The carbohydrate-free F-pili are "lighter" in weight than their "complex" counterparts. No evidence, thus far, has suggested the presence of any large amount of contaminant material in "pure" F-pili preparations. Final chemical analysis of purified material is described in the next chapter.

CHAPTER VI

BIOCHEMICAL COMPOSITION OF F-PILI

To date, almost all that is known about the chemical nature of F-pili has been derived in an indirect manner. For example, it has been shown that these filaments are disrupted by exposure to organic solvents such as chloroform, benzene, and carbon tetrachloride (Wendt et al., 1966), to the proteolytic enzymes papain, trypsin, chymotrypsin and pepsin (Brinton and Beer, 1967), and to brief exposure to 80° temperatures (Valentine et al., 1969). The above evidence, plus the fact that F-pili form side-to-side aggregates with themselves in aqueous solutions suggests that F-pili are high in protein content. Bayer (1968a-c) has indicated that the F-pilus appears functionally as an extension of a bacterial wall-membrane complex with specialized receptors for male-specific phages. It is possible, therefore, that lipid and/or carbohydrate may be associated with purified experimental preparations. The fact that different antigenic types of F-pili have been isolated (Nishimura et al., 1967) is, alone, sufficient evidence to suspect the involvement of carbohydrate in the F-pilus structure.

To probe these, and several other, unknown areas of F-pili chemistry, the following pilot study was undertaken. It is hoped that this study, although incomplete, will further our understanding of the biology of these organelles.

A. RESULTS

(1) Protein Analysis

The approximate protein content of F-pili material was determined

on a routine basis using the spectrophotometric method of Lowry et al. (1951) with bovine serum albumin as standard. Absolute quantitation of the weight percent of protein in "pure" F-pili material was not attempted at this time due to a lack of a true reference standard and because variable amounts of carbohydrate were found associated with "pure" F-pili preparations (see section A(2), below).

To obtain a partial amino acid analysis of F-pili protein, it was first necessary to prepare F-pilin free of conjugate carbohydrate material. This was achieved by prehydrolysis of intact, "purified" F-pili preparations in 10% TCA for 3 hours at 100°. F-pilin prepared in this manner was found to contain no apparent carbohydrate material, as determined by reaction to orcinol-HCl (see section A(2), below). The denatured F-pilin was collected from suspension by differential centrifugation at 10,000 x g for 20 minutes, washed 3 times with distilled-deionized water, and lyophilized to dryness. Eight hundred to 1000 µg of such preparations were hydrolyzed in 6.7 N HCl at 110° for 24 hours, and a partial amino acid analysis of the resultant mixture obtained by the methodology given in Chapter II. Results of this procedure are presented in Table 6.1.

Amino acid analysis of F-pilin revealed that aspartic and glutamic acid content was particularly high in the experimental material. About 22% of the total amino acid composition of the F-pilin examined was collectively contributed by these two amino acids alone. Cystine, cysteine and methionine were converted to cysteic acid and methionine sulfone, respectively, by performic oxidation before hydrolysis. The values of serine and threonine were back calculated to original content assuming 10.5% decomposition of original serine content and 5.3% decomposition of

Table 6.1

Partial Amino Acid Composition of F-Pilin

Amino Acid	Micromoles Amino Acid/ 100 Micromoles Protein Hydrolysate
Lysine	5.63
Histidine	1.44
Arginine	4.86
Aspartic Acid	10.29
Threonine	5.18
Serine	4.85
Glutamic Acid	12.03
Proline	4.77
Glycine	9.22
Alanine	9.62
Valine	8.37
Methionine	2.23
Isoleucine	5.81
Leucine	8.00
Tyrosine	2.51
Phenylalanine	4.15
Cysteic Acid	1.04

original threonine content after 24 hours of hydrolysis in 6 N HCl at 110° (Eastoe, 1966). The above corrections for serine and threonine, however, must be considered approximate in light of the fact that amino acid units in peptide combination may be more or less prone to decomposition than those in the free state (Eastoe, 1966). As tryptophane content did not enter into the tabulation of the molar ratios of amino acids present per 100 μmoles of protein hydrolysate, all values should, at present, be considered approximations of the true values contained in F-pilin.

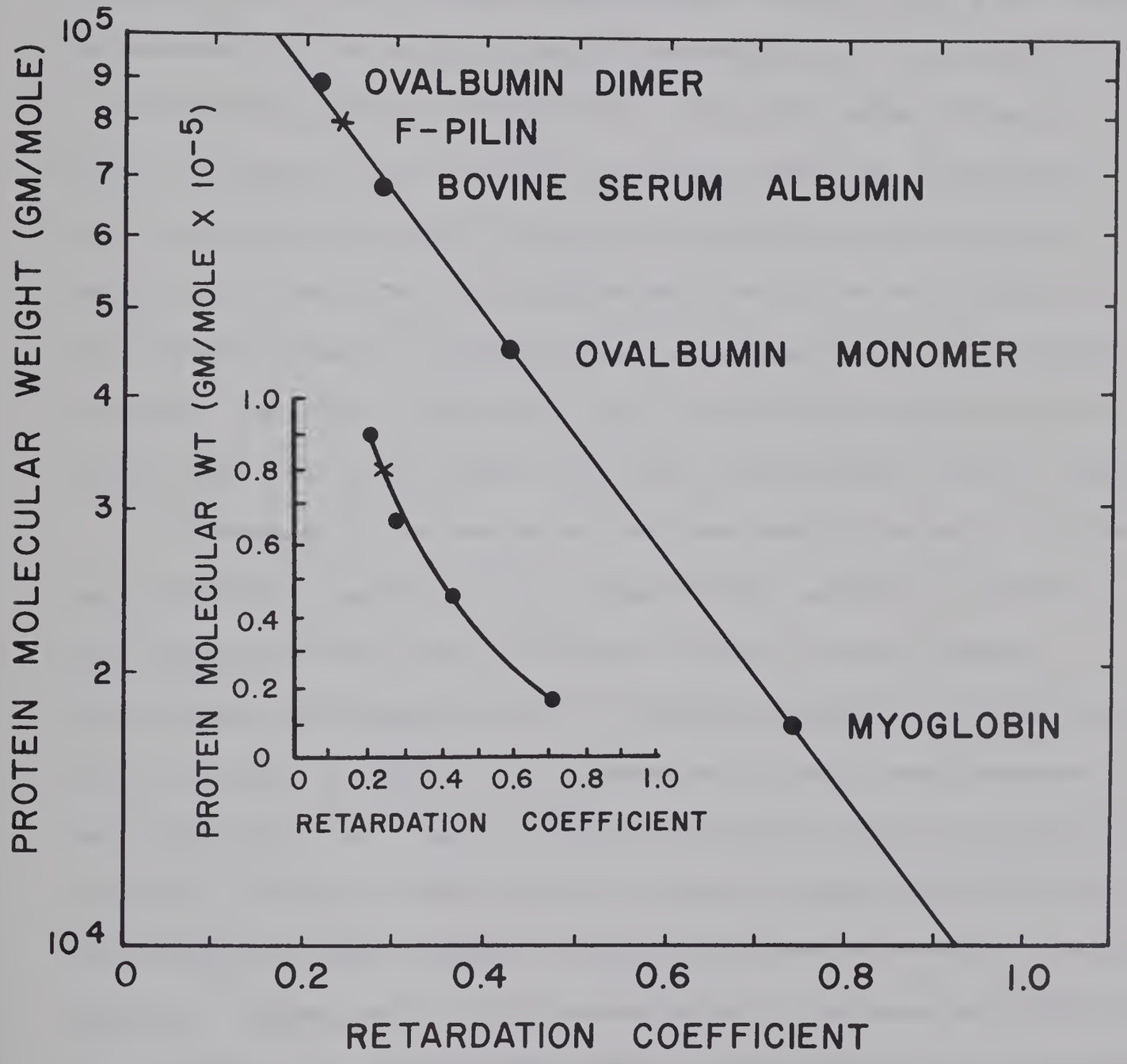
To determine an approximate molecular weight of F-pilin subunits, complexes of F-pili protein were made with the anionic detergent sodium dodecyl sulfate (SDS), and the SDS-complexed material electrophoresed in 10% SDS-polyacrylamide gels, pH 7.1 (see Chapter II for details). After electrophoresis, experimental gels were stained for protein with Coomassie Blue, revealing two hypersharp bands coelectrophoresing together (Figures 5.4, 5.5). The stained F-pilin components were shown to possess an inherent average molecular weight of 8×10^4 daltons as measured by relative retardation in the electrophoretic gels. Extrapolation of the estimated molecular weight of F-pilin was made from curves of standard proteins whose molecular weights and relative gel positions were known (Figure 6.1).

(2) Carbohydrate Analysis

Initial investigation of the carbohydrate content of F-pili preparations began by incubating microlitre quantities of "purified" experimental material with the anthrone-sulfuric acid reagent. This reagent was used as a qualitative indicator of carbohydrate content in F-pili samples because colors can be developed with hexoses, pentoses, deoxy

Figure 6.1. Molecular weight determination of subunit F-pilin using a 10% polyacrylamide gel containing 0.1% SDS.

The relative mobility of each standard protein (●) was plotted against its apparent molecular weight. The apparent molecular weight of subunit F-pilin (X) was then determined by its electrophoretic mobility with the aid of this plot. A rectilinear plot of the same data is provided in the insert.


PROTEIN MOLECULAR WEIGHT (GM/MOLE)
10^5
9
8
7
6
5
4
3
2
10^4
OVALBUMIN DIMER
F-PILIN
BOVINE SERUM ALBUMIN
OVALBUMIN MONOMER
MYOGLOBIN
0
0.2
0.4
0.6
0.8
1.0
RETARDATION COEFFICIENT
PROTEIN MOLECULAR WT (GM/MOLE X 10^{-5})
1.0
0.8
0.6
0.4
0.2
0
0 0.2 0.4 0.6 0.8 1.0
RETARDATION COEFFICIENT

sugars, hexuloses, uronic acids, heptuloses, hexose and hexulose phosphates, alditols, and 6-deoxyhexoses (Aminoff et al., 1970). F-pili preparations reacted very strongly with the anthrone-sulfuric acid reagent, producing dark green solutions which were read for absorption at 620 mμ. The total estimated carbohydrate content of any given sample, as measured by this reaction, was all recoverable in later assays as orcinol-reacting material (Table 6.2). The higher values of sugar content obtained by the orcinol reaction (in comparison to anthrone) were not unexpected, since the anthrone reaction is one of several general color reactions of carbohydrates in which the sensitivity toward various classes of carbohydrates, e.g., mono- and polysaccharides, is of the same order of magnitude, and the absorption maxima are very nearly identical for more than one class of carbohydrate (Dische, 1962).

To determine if pentose sugar, and thus possibly nucleic acid, was associated with "purified" F-pili preparations, analysis of "pure" F-pili concentrates was made by reaction to Bial's orcinol reagent. Short heating (20 minutes at 100°) of F-pili material with a 0.2% solution of orcinol in 30% HCl, in the presence of ferric ions, produced dark green solutions (plus precipitates) which were read for absorption at 650 mμ. Positive identification of pentose content in F-pili material could not be made, however, because 6-deoxyhexose, hexuronic acids, heptoses, trioses, and, in high concentrations, D-mannose and D-galactose also produce a green color under identical conditions with an absorption maxima ranging between 650 and 670 mμ (Dische, 1962). Further colorimetric and chromatographic analysis (below) eliminated ribose and deoxyribose as possible chemical constituents of the F-pili material tested.

To more accurately identify the class(es) of carbohydrate(s) and

Table 6.2

Estimates of the Carbohydrate Content of "Purified" F-Pili Material by the Anthrone-H_2SO_4 and Orcinol-HCl Color Reactions

10 to 100 µl quantities of a "purified" F-pili concentrate were incubated with the appropriate color reagent at 100° according to the methodology referred to in Chapter II. Absorbance of final solutions was read in a Beckman DU spectrophotometer at the wavelengths described in the text. Results were expressed as the mg equivalents of ribose and/or adenosine ribose present per ml of F-pili suspension.

Reaction	Total Volume F-Pili Suspension	F-Pili Conc./ml of Suspension	Amount of Carbohydrate/ml
Anthrone-H_2SO_4	30.0	2.3×10^9 CE	2.71 mg (ribose std.)
Orcinol-HCl	30.0	2.3×10^9 CE	7.87 mg (adenosine ribose std.)

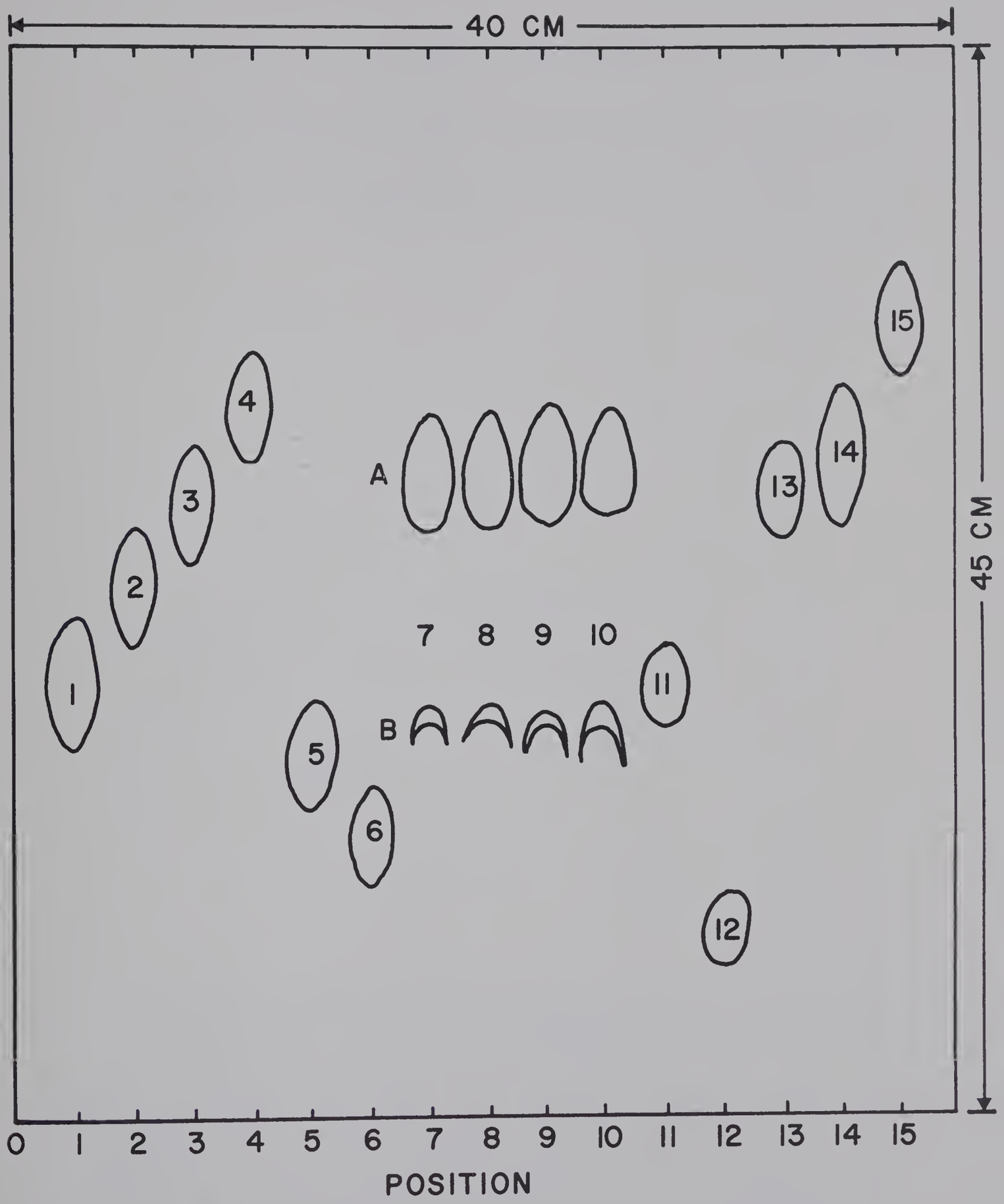
individual sugar(s) present in F-pili preparations, the carbohydrate material of "pure" F-pili material was deproteinized by trichlorotrifluoroethane treatment (see Chapter II), and the resultant sugar material subjected to mild acid hydrolysis at 100° to convert oligo- and polysaccharide compounds to the monosaccharide state. No detectable protein material was found associated with such preparations, as analyzed for by the Lowry reaction. The resultant sugar mixture was then separated by paper chromatography (see Chapter II for details). One major (A) and one minor (B) component were consistently observed (Figure 6.2). The major spot corresponded in approximate position to both the aldose pentose sugars, xylose and lyxose, and the 6-deoxyhexose sugar, fucose, and comprised greater than 90% of the unknown sugar material. The minor spot migrated identically with the aldose sugar, galactose. When the major and minor sugar components were eluted from paper, concentrated to dryness, and reheated in 0.48 N HNO_3 at 100° for 1 hour, the same chromatographic pattern of unknowns was obtained as was apparent in the original hydrolyzate (Figure 6.3). This indicated that the hydrolysis procedure used to convert the unknown sugars to monosaccharides was complete. Both the major and minor components of sugar hydrolyzates, when eluted from chromatographs, gave positive orcinol and anthrone reactions when tested. Both components were identified as reducing sugars by their strong reaction to the ammoniacal-$AgNO_3$ and aniline-phosphoric acid sprays of Krebs et al., 1969 (see Chapter II). The reductive property of these unknown sugars was qualitatively confirmed by positive reaction of both components with Benedict's solution. 2-Deoxypentose content in the experimental preparations, as analyzed for by reaction to the Dische diphenylamine reagent (with deoxyadenosine

Figure 6.2. Paper chromatographic separation of acid-hydrolyzed F-pili - associated carbohydrate.

Separation of standard and unknown sugars was made on Whatman No. 1 chromatography paper utilizing 1-butanol:pyridine:water (6:4:1) as the developing solvent. 18-hour runs were made on 40 x 45 cm serrated-edged chromatographs. Standard sugars (100 µg each) employed were as listed below. Relative mobilities of standard and unknown sugars were:

Position	Sugar	R_{ribose}
1	Glucose	0.66
2	Arabinose	0.77
3	Xylose	0.88
4	Ribose	1.00
5	Galactose	0.55
6	Sucrose	0.43
7-10	F-pili hydrolyzate	
7	10 µl	0.92 (A) 0.54 (B)
8	20 µl	0.92 (A) 0.54 (B)
9	30 µl	0.93 (A) 0.53 (B)
10	40 µl	0.93 (A) 0.55 (B)
11	Glucosamine	0.62
12	Galactosamine	0.29
13	Fucose	0.88
14	Lyxose	0.96
15	Rhamnose	1.12

The hydrolyzate of unknown carbohydrate material examined contained the equivalent of 20 mg ribose/ml of solution. Scale of chromatograph 1:2.5. Detection spray was ammoniacal-$AgNO_3$.


40 CM
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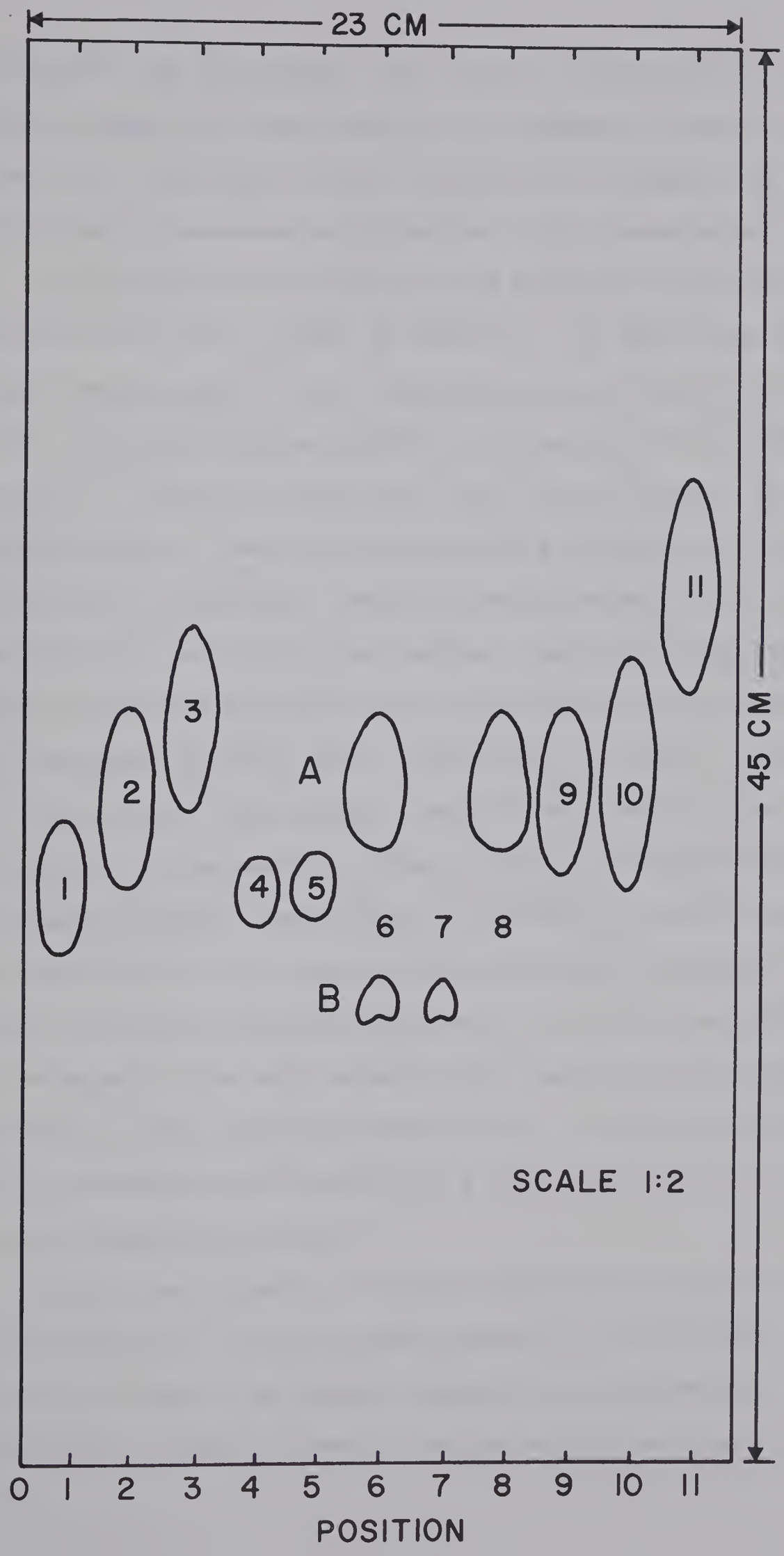
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
POSITION

Figure 6.3. Paper chromatographic separation of twice acid-hydrolyzed F-pili-associated carbohydrate.

Separation of standard and unknown sugars was made on Whatman No. 1 chromatography paper utilizing 1-butanol:pyridine:water (6:4:1) as the developing solvent. 18-hour runs were made on 23 x 45 cm serrated-edged chromatographs. Standard sugars (100 µg each) employed were as listed below. Relative mobilities of standard and unknown sugars were:

Position	Sugar	R_{ribose}	
1	Arabinose	0.77	
2	Xylose	0.90	
3	Ribose	1.00	
4-5	0.48 N HNO_3		
4	20 µl	0.72	
5	40 µl	0.73	
6-8	F-pili hydrolyzate		
6	original 20 µl	0.90 (A)	0.58 (B)
7	2x hydrolyzed B		
	20 µl	-	0.58
8	2x hydrolyzed A		
	20 µl	0.90	-
9	Fucose	0.90	
10	Lyxose	0.97	
11	Rhamnose	1.18	

Components A and B, obtained from original sugar hydrolyzates by preparative paper chromatography, were eluted from preparative chromatographs, taken to dryness under vacuo, reconstituted in 0.48 N HNO_3 to their original concentration, and reheated at 100° for 1 hour. The experimental sugars were then rechromatographed along with the original hydrolyzate to determine the extent of sugar hydrolysis to monosaccharides. A nitric acid control was also chromatographed. The original hydrolyzate of unknown carbohydrate material (position 6) contained the equivalent of 20 mg ribose/ml of solution. Scale of chromatograph 1:2. Detection spray was ammoniacal-$AgNO_3$.


23 CM
45 CM
1
2
3
A
4
5
6
7
8
9
10
11
B
SCALE 1:2
0 1 2 3 4 5 6 7 8 9 10 11
POSITION

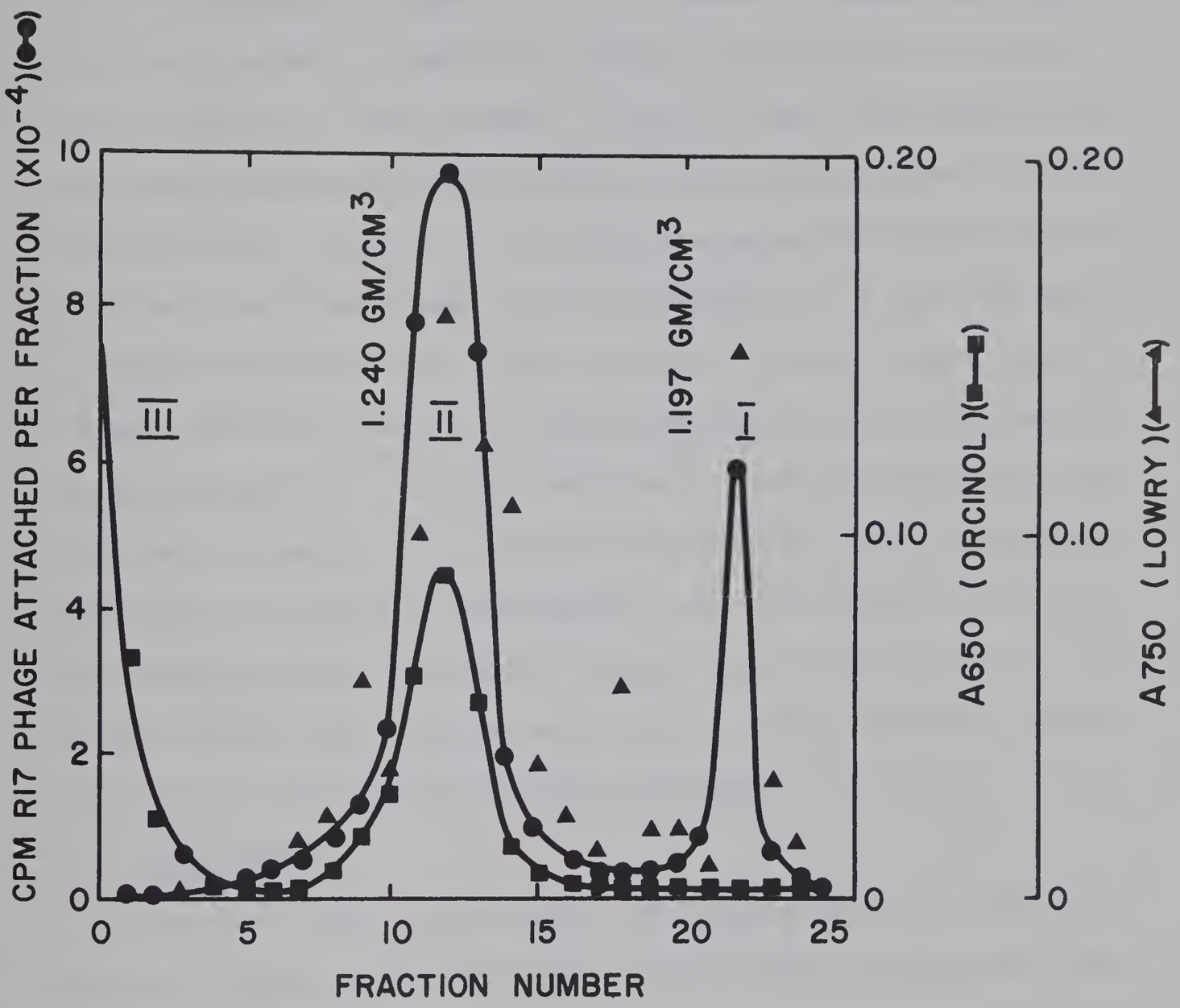
as standard), was non-existent. The aldose or ketose nature of the component sugars was, unfortunately, not determined. However, recent evidence (R. O'Callaghan, personal communication) suggests that ketose sugar(s) may be associated with "purified" F-pili preparations.

The distribution of orcinol-reacting material in a CsCl-banded preparation of F-pili is shown in Figure 6.4. It may be seen that orcinol-reacting material was found associated with "heavy" pili (ρ = 1.240 - 1.245 g/cm^3) and as a pellet at the bottom of the gradient (peak III). Subsequent studies have shown that the bulk of the carbohydrate in peak III does not originate with F pili but is a contaminant arising from a carbohydrate component normally present in the tryptic case soy broth used as the growth medium. That this carbohydrate component arising from the medium is not responsible for the presence of the carbohydrate moiety in peak II was shown in two ways. Firstly, it was found by paper chromatographic analysis that "pellet" (peak III) carbohydrate is qualitatively different from peak II carbohydrate (R. O'Callaghan, personal communication). Secondly, it has been found that CsCl gradients of F-pili prepared from cells grown in synthetic medium contain carbohydrate associated with peak II, but lack the broth-specific carbohydrate that would normally form a pellet at the bottom of the gradient (L. Frost, personal communication). It was thus concluded that peak II carbohydrate most probably is a true component of F-pili rather than a contaminating substance.

An additional property of F-pili, observed as a result of banding in CsCl gradients, is that prolonged exposure to the high CsCl environment (1.8 M) leads to an apparent dissociation of carbohydrate and protein moieties in peak II material, suggesting that the sugar and protein

Figure 6.4. Phage-attachment, total carbohydrate, and total protein profiles of isopycnically-banded "pure" F-pili material, obtained from bacterial cultures grown in TSB.

CsCl density gradients of "pure" F-pili material, prepared from "crude" preparations according to the methodology outlined in the text, were collected in 12 drop fractions and then diluted to 2.0 ml with basic TMM. Each fraction was divided in half, and to one portion was added 1.0 ml basic TMM containing 2-ME (final concentration = 12 mM), $MgCl_2$ (final concentration = 10 mM), and 5 x 10^{12} particles ^{32}P-labeled R17 phage (2 x 10^6 c.p.m.). Phage attachment was allowed to proceed for 40 minutes at 4°, after which time appropriate fractions were assayed for attached c.p.m., as described in Chapter II. To the second portion of each original fraction was again added 1.0 ml basic TMM. These diluted samples were dialyzed of CsCl and then divided in half, one portion serving for semi-quantitative detection of carbohydrate (reaction to orcinol-HCl), the second for semi-quantitative detection of protein (Lowry procedure). The buoyant density (25°) of peaks I and II was ascertained from refractive index measurements of undiluted fractions of the original gradient (not shown).


CPM R17 PHAGE ATTACHED PER FRACTION (X10⁻⁴)(●—●)
A650 (ORCINOL)(■—■)
A750 (LOWRY)(▲—▲)
1.240 GM/CM³
1.197 GM/CM³
FRACTION NUMBER
0
2
4
6
8
10
0.10
0.20
5
15
20
25

components may be held together by electrostatic forces. When peak II F-pili were dialyzed against 1000 volumes of basic TMM containing 2-ME (final concentration = 6 mM) at 4° for 20 hours to remove the cesium salt, and rebanded in fresh CsCl gradients, patterns such as the one shown in Figure 6.5 were obtained. As may be seen, both sugar and protein became separated from one another with concomitant loss of F-pili phage-attachment capacity. This occurrence suggested that the variable position of once-banded peak II F-pili material (ρ = 1.240-1.245 gm/cm^3) in normal density gradients might be due to a variable amount of association/dissociation (at 4°) of F-pili carbohydrate with F-pili protein the exact amount of F-pili-associated sugar found being dependent upon the time of exposure to the high salt environment. This would perhaps explain why the amount of carbohydrate found associated with "pure" F-pili material varied considerably from one preparation to the next, and it was for this reason that we were unable to obtain an exact value for the ratio of carbohydrate to protein (by weight) in F-pili.

(3) Lipid Analysis

"Purified" F-pili concentrates were analyzed for total lipid content, as follows. One-ml aliquots of "pure" F-pili concentrates were desalted by extensive dialysis against distilled-deionized water, and the dialysate then taken to dryness under vacuo. This dehydrated material was then thrice extracted with 17 ml volumes of $CHCl_3$:CH_3OH (3:2) each, and the pooled extracts reduced to a volume of 1 ml under vacuum. The concentrated extract was analyzed for lipid content by thin-layer chromatography on Silica Gel H plates of uniform 250 μ thickness. Neutral lipids were separated in the solvent system petroleum ether: diethyl ether: glacial acetic acid (85:15:1). Phospholipids were like-

Figure 6.5. Protein, sugar and phage-attachment profiles of twice-banded peak II F-pili in a normal 1.8 M CsCl density gradient.

Once-purified, salt-free complexes of peak II F-pili (see Figure 6.4) were rebanded in 1.8 M CsCl gradients as described in Chapter II, and 10-drop fractions collected. Refractive index measurements at 25° were used to calculate the buoyant density of each undiluted fraction. All fractions were then diluted to a volume of 2.0 ml with basic TMM. To one-half of each original fraction was added 1.0 ml of basic TMM containing 2-ME (final concentration = 12 mM), $MgCl_2$ (final concentration = 10 mM), and 5.0×10^{12} particles ^{32}P-labeled R17 phage (2×10^6 c.p.m.). Phage attachment was allowed to proceed for 40 minutes at 4°, after which time the c.p.m. attached per fraction were determined. The remaining portions of the original gradient were dialyzed of CsCl, and equal volumes used for carbohydrate and protein analyses, ribose and bovine serum albumin serving as standards.

CARBOHYDRATE / PROTEIN RATIO (▲—▲)
BOUYANT DENSITY IN CsCl at 25° (GM/CC)(●—●)
CPM 32P-LABELED RI7 PHAGE ATTACHED PER FRACTION (X10-2)(■—■)
FRACTION NO.
0
10
20
30
40
50
60
1.150
1.200
1.250
1.300
1.350
1.400
0
1
2
3
5
10
15
20
25
30

wise separated in the solvent system $CHCl_3$:CH_3OH:NH_4OH:H_2O (65:30:2:3). Visualization of all lipid components was performed by spraying the plates with 60% (aq.) H_2SO_4, and charring at 110° for 30 minutes. Within the limits of detection by this system, "purified" F-pili were found to contain < 1 μg of lipid per 200 μg of F-pili protein. Since the amount of protein in F-pili was found to represent less than one-half of the total F-pili mass (the main component being carbohydrate in nature), results from the lipid analysis were taken to mean that F-pili are essentially lipid free. I am indebted to Mr. J. S. Chen for performing these analyses.

(4) Nucleic Acid Analysis

Since chromatographic and colorimetric analyses carried out earlier (Section A(2), above) had shown ribose and deoxyribose to be absent in "pure" F-pili material, it was evident that F-pili were probably free of nucleic acids. Additional support for this view was provided by an analysis of the phosphate content in F-pili.

It was reasoned that if nucleic acid was present in "pure" F-pili material, one should find, before and after hot 5% TCA hydrolysis, the same molar ratios of TCA-insoluble carbohydrate and phosphate as are present in native DNA or RNA (i.e., 1/1 before hydrolysis and 0 after hydrolysis). To see if "pure" F-pili material possessed the necessary quantities of carbohydrate and organic phosphate to contain nucleic acid, and if the sugar and phosphate behaved in a nucleic acid-like manner, the following experiment was performed.

Various aliquots (0.10 to 0.50 ml) of a "pure", desalted F-pili concentrate in a total volume of 1.0 ml aqueous solution was analyzed

for total sugar (orcinol-HCl) and total phosphate content, as described in Chapter II. (Ribose and KH_2PO_4 served as standards in these analyses). Identical amounts of F-pili material were made 5% with respect to TCA and heated at 90° for 45 minutes. After 45 minutes of heating had elapsed, all solutions were cooled to 4° in an ice bath and the precipitated material harvested by differential centrifugation (4°) at 8000 x g for 10 minutes. The aqueous phase was removed by aspiration, and the pellet material washed 3 times with 1.0 ml aliquots of cold 5% TCA. The pellet material was then resuspenced in 1.0 ml distilled-deionized water, vigorously shaken for 3 minutes by vortex mixing, and analyzed for total sugar and total phosphate content. Results were expressed in terms of the ratio of carbohydrate to phosphate recovered from the entire F-pili concentrate, and are shown in Table 6.3.

From the results presented in Table 6.3, it can be seen that "pure" F-pili material possessed far too little phosphate to contain nucleic acid; ratios of 196 moles carbohydrate (based on a ribose standard) to 1 mole KH_2PO_4 were obtained for untreated F-pili material, whereas identical preparations hydrolyzed with hot 5% TCA possessed a ratio of 87.9 moles carbohydrate to 1 mole KH_2PO_4. This drop in carbohydrate to KH_2PO_4 ratio was attributed to sugar hydrolysis, as equal amounts of TCA-insoluble phosphate were found before and after TCA hydrolysis, suggesting that the phosphate was protein-linked.

B. SUMMARY AND DISCUSSION

Results of this chapter may be summarized as follows. Biochemical analyses of "pure" F-pili material have shown carbohydrate, protein and phosphate to be associated with experimental preparations, the F-pili

Table 6.3

Total Sugar and Total Phosphate Content
of a "Pure" F-Pili Preparation

Preparation	Total µg "Ribose" Recovered	Total µg "KH_2PO_4" Recovered	$\frac{\text{Carbohydrate}}{KH_2PO_4}$
"Pure" F-pili (untreated)	5.98×10^4	338	$\frac{196}{1}$ (molar)
"Pure" F-pili (after TCA hydrolysis)	2.69×10^4	338	$\frac{87.9}{1}$ (molar)

examined being devoid of lipid and nucleic acid. The protein constituent of F-pili, F-pilin, was shown to possess an inherent average subunit molecular weight of 8×10^4 daltons, as measured by its relative retardation in 10% SDS-polyacrylamide gels, pH 7.1. Amino acid analysis revealed that about 22% of all the amino acids present in F-pilin are collectively represented by aspartic and glutamic acids. Carbohydrate analysis showed that two unknown reducing sugars were present in "pure" F-pili concentrates, these sugars most probably being a hexose (sugar B, Figure 6.2) and a pentose or deoxyhexose (sugar A, Figure 6.2) in nature. Ribose and deoxyribose were not present in experimental preparations. The organic phosphate content of "pure" F-pili material was shown not to be phospholipid or nucleic acid in nature.

Although the results from the foregoing experiments do not permit exact quantitation of all the components present in the experimental material examined, it was found that F-pili might contain as much as 90% (by weight) carbohydrate, with protein being present in a concentration of 5-10%, and organic phosphate at about 1%. However, the true distribution of chemical constituents could be significantly different from these amounts since it was found to be virtually impossible to obtain "pure" F-pili by means of CsCl density gradient centrifugation which had a reproducible ratio of carbohydrate to protein (by weight) or carbohydrate to phosphate (by weight). The significance of the distribution of chemical constituents in F-pili is not understood at present, although it is possible that F-pili may be related to either peptidoglycan or glycoprotein material. Further investigation of the protein-carbohydrate-phosphate nature of F-pili is needed to elucidate the obvious chemical complexity of this bacterial appendage.

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